THE ROLE OF THE BETAINE/GABA TRANSPORTER IN EPILEPSY, SEIZURE SUSCEPTIBILITY, AND BEHAVIOR

by

Nicole M. Rowley

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STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Nicole M. Rowley**

has been approved by the following supervisory committee members:

H. Steve White	, Chair	**12/10/2010** Date Approved
Karen Wilcox	, Member	**12/10/2010** Date Approved
Annette Fleckenstein	, Member	**12/10/2010** Date Approved
J. Gregory Lamb	, Member	**12/10/2010** Date Approved
Arne Schousboe	, Member	**12/10/2010** Date Approved

and by **William Crowley** , Chair of

the Department of **Pharmacology and Toxicology**

and by Charles A. Wight, Dean of The Graduate School.

ABSTRACT

Epilepsy is a chronic neurological disorder characterized by the presence of unprovoked, recurrent, spontaneous seizures. The GABA hypothesis of epilepsy states that a decrease in GABAergic tone leads to an imbalance between excitatory and inhibitory transmission and increases seizure susceptibility. The extracellular levels of GABA are controlled by the GABA transporters (GATs), including the betaine/GABA transporter (BGT1). BGT1 transports both GABA and the organic osmolyte, betaine. Inhibitory tone and osmotic balance both play roles in controlling neuronal excitability, making BGT1 a potential target for the modulation of excitability. Recently, studies utilizing an equipotent inhibitor of GAT1 and BGT1, EF1502, and TGB, a GAT1 selective inhibitor, suggested that BGT1 inhibition is anticonvulsant.

Seizures, pain, anxiety, depression, and other neuropsychiatric disorders are influenced by the GABAergic system, including modulation by GAT activity. However, little is known about the involvement of BGT1. Since BGT1 may be involved in the control of osmotic balance and extracellular GABA levels, it has the potential to be involved in the modulation of these disorders. The current dissertation was designed to test the hypothesis that BGT1 expression plays a role in epilepsy, seizure susceptibility, and other neurological disorders, including depression, anxiety, pain, and cognition, as well as motor function.

To test the hypothesis that BGT1 expression is involved in epilepsy, a model of temporal lobe epilepsy, i.e., status epilepticus (SE), was utilized and BGT1 mRNA expression determined at several time-points following SE. BGT1 mRNA expression was compared to that of the other GATs and osmoprotective genes. From these studies, it appears that the expression of BGT1 may be involved in the pathogenesis of epilepsy. To test whether BGT1 is involved in the regulation of acute seizure susceptibility, four models of seizure threshold were utilized in BGT1 KO and WT mice (minimal tonic extension, minimal clonic, 6 Hz, and i.v.PTZ seizure threshold tests). Furthermore, the rate of corneal kindling acquisition was ascertained. No differences in seizure threshold were observed between genotypes. However, BGT1 KO mice displayed a behavioral profile distinct from that of their WT littermate controls. BGT1 KO mice have increased depressive and anxiety-like behaviors as well as a 'manic' phenotype. The results from this dissertation provide evidence in support of continued investigation of BGT1 in a number of distinct CNS disorders.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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CHAPTER 1

INTRODUCTION

Epilepsy: a clinical and preclinical challenge

Epilepsy is a chronic neurological disorder characterized by the presence of unprovoked, recurrent, spontaneous seizures. Seizures are symptoms of excessive, synchronous neuronal firing. According to the Epilepsy Foundation of America (2005), more than 3 million people in the US have epilepsy, and 200,000 new cases are diagnosed each year, incurring an estimated annual cost of $15.5 billion. Despite many years of research, epilepsy remains an ongoing clinical challenge. One of the difficulties in treating epilepsy is the heterogeneity of the disorder; more than 40 different types of epilepsies and seizure disorders exist. Another factor is that the underlying cause of seizures is unknown in 70% of cases (Epilepsy Foundation of America, 2005). A third factor contributing to the challenge of epilepsy treatment is that only 50% of patients respond to the first antiepileptic drug (AED) they are prescribed, and only one-third of the remaining population responds to the second AED (Kwan and Brodie 2000). The response rate drops precipitously with further medication, resulting in pharmacoresistance in approximately 30% of epilepsy patients. If a patient fails to respond to the first AED administered, they only have a 10-15% chance of

responding to any subsequent AED (Loscher 2006). The result is an unacceptably large proportion of patients who are not adequately treated by currently available therapies. Unfortunately, the approval of several new AEDs since 1993 has not significantly altered the rate of pharmacoresistance, although the pharmacokinetic, safety and side-effect profiles have improved substantially. The ongoing challenge of seizure control highlights the need for new AEDs with novel mechanisms of action.

The systemic approach to anticonvulsant drug discovery began in 1937 with the discovery of phenytoin using the maximal electroshock model (Putnam and Merritt 1937). Since then, several preclinical models have been developed and employed in AED discovery (White et al. 2002a). Nonetheless, none of the currently available animal models can accurately predict clinical efficacy for every compound. Therefore, adequate preclinical testing of a compound requires the utilization of several preclinical models that represent different seizure types. Each model that was utilized in this dissertation is explained in greater detail in the “Mouse models” section of Chapter 1 and in Chapter 3.

GABA and GABA transporters

GABA: γ-aminobutyric acid (GABA) is recognized as the predominant inhibitory neurotransmitter in the mammalian brain regulating network exciteability (Mody and Pearce 2004). GABA is released from synaptic vesicles and acts upon ionotropic $GABA_A$ receptors to induce a hyperpolarizing chloride current. It also activates metabotropic $GABA_B$ receptors that induce potassium

efflux to hyperpolarize the neuron (Feldman et al. 1997; Watanabe et al. 2002). GABA's action is terminated by diffusion and re-uptake by GABA transporters (GATs). While approximately 80% of released GABA is transported back into the presynaptic terminal where it is repackaged for release, the remainder is transported into the surrounding astrocytes. Here, GABA is converted via GABA-transaminase and succinic semialdehyde dehydrogenase to succinate, which enters the tricarboxylic acid cycle. Succinate may be metabolized into CO_2 or converted to α-ketoglutarate and subsequently to glutamine, which can be shuttled back to the presynaptic terminal. In the presynaptic terminal, glutamine is converted to glutamate, which is then converted into GABA by glutamate decarboxylase (GAD); the presence of GAD is the hallmark marker of GABAergic neurons (Hertz and Schousboe 1987; Gram et al. 1988; Kugler 1993; Feldman et al. 1997).

GABA transporters: GABA transport is dependent on four transporters, solute carrier (SLC) 6a1, SLC6a12, SLC6a13, and SLC6a11, termed GAT1-4 (mice) or GAT1, BGT1, and GAT2 and 3 (rat and human). The rat/human nomenclature is suggested by the HUGO Gene Nomenclature Committee and will be used throughout the remainder of this dissertation.

Structure and function: The GABA transporters belong to the SLC6 family of transporters, which contain 12 transmembrane domains (TMDs) and function as dimers (Beleboni et al. 2004). Both the N- and C- termini are intracellular and contain phosphorylation sites that function to regulate transport. TMD 1, intracellular loop (IL) 2, and extracellular loops (EL) 2 and 3 are integral for

tertiary structure stabilization, and EL 4-6 are believed to be involved in substrate binding (Jursky et al. 1994; Kanner 1994; Tamura et al. 1995). Sequence alterations in these ELs lead to mutants with predictable transport kinetics and substrate specificity. For example, substituting the sequence for GAT2 into EL6 of GAT1 leads to a K_m of GABA similar to that of GAT2. Substituting the GAT3 sequence into EL5 of GAT1 enables the transport of β-alanine through the mutant, while native GAT1 cannot transport β-alanine (Tamura et al. 1995). Site-directed mutagenesis has found five residues essential for transport activity (R69, W68, E101, Y140, and W222), and tyrosine-140 is also imperative for GABA binding. Transport requires minimal lengths of IL4 and EL4 (Bismuth et al. 1997; Schousboe and Kanner 2002).

SLC6 transporters are Na^+ and Cl^- dependent, and are driven by the electrical gradient of Na^+. GAT1 transports 2 Na^+ and 1 Cl^-, while BGT1 transports 3 Na^+ and 1 Cl^- with each molecule of substrate (Matskevitch et al. 1999). The GATs are able to maintain a large gradient (10^5) between intra- and extracellular GABA (Beleboni et al. 2004). Since the transporters are driven by an electrogenic mechanism, they are able to reverse transport under certain conditions. When the membrane potential is more negative than the reversal potential of the transporter, it will function in the forward (inward) direction, while it will function in reverse if the membrane potential is above the reversal potential. Due to this, an increase in intracellular Na^+ or strong depolarization can reverse GATs (Richerson and Wu 2003). The ability of GATs to reverse and release GABA into the extracellular environment may be important in seizure cessation or in

preventing seizure spread (Patrylo et al. 2001). Patients with epilepsy have reduced seizure-induced increases in extracellular GABA, perhaps due to the downregulation of GATs (During et al. 1995).

Localization: GAT1: GAT1 is abundant in the brain and displays a wide distribution pattern that is consistent with the labeling of GABAergic neurons. It is found in high levels in the retina, olfactory bulb, ventral pallidum, interpeduncular nucleus, cortex, hippocampus, and cerebellum (Durkin et al. 1995; Borden 1996). GAT1 is absent on cell bodies: it is located on axonal segments of presynaptic GABAergic neurons where it is integral for the presynaptic reuptake of released GABA (Radian et al. 1990; Borden 1996; Conti et al. 1998).

BGT1: Mouse BGT1 was originally cloned and referred to as GAT2. It was determined to be the mouse homologue to canine and human BGT1 (88% and 87% sequence homology, respectively) (Lopez-Corcuera et al. 1992; Borden et al. 1995a). Mouse GAT2 is therefore termed BGT1 in this report as suggested by the HUGO Gene Nomenclature Committee. BGT1 mRNA is widely distributed throughout the brain (Lopez-Corcuera et al. 1992; Borden et al. 1995a). However, BGT1 protein expression patterns *in vivo* are currently unvalidated, in part due to the absence of knockout data and to questionable antibody specificity. Using available antibodies, BGT1 was reported to be expressed on neurons, and to be located extrasynaptically, but not necessarily in close proximity to GABAergic synapses (Zhu and Ong 2004b). BGT1 has been localized mainly to astrocytes based on *in vitro* mRNA and pharmacological studies (Borden et al. 1995a; Borden et al. 1995b; Borden 1996). However, *in*

situ hybridization studies suggest mostly neuronal expression (Borden 1996). BGT1 and GAT1 are expected to be differentially localized due to their basolateral and apical expression, respectively, in polarized epithelial cells (Ahn et al. 1996). The basolateral expression of BGT1 suggests either a post- or extra-synaptic action (Borden 1996). *In situ* hybridization studies and microinjection of cDNA into cultured hippocampal cells suggest that BGT1 is not localized exclusively to GABAergic synapses (Borden et al. 1995a; Ahn et al. 1996). Due to the supposed extrasynaptic location of BGT1, it is not likely to be contributing to the termination of fast, phasic neurotransmission. It is more likely playing a role in tonic inhibition (Mody 2001). It has also been suggested that BGT1 is not functionally important as a GABA transporter, but that its major role in the brain is to serve as an osmolyte transporter.

GAT2: GAT2 is expressed abundantly in the neonatal brain and at very low levels in the mature brain. It is found in both neurons and astrocytes, primarily extrasynaptically. GAT2 is also found in the leptomeninges, and is believed to play a role in the regulation of cerebrospinal fluid GABA levels (Durkin et al. 1995; Borden 1996; Conti et al. 1999).

GAT3: GAT3 is abundantly expressed and widely distributed in the brain, albeit less so than GAT1. Although it is expressed in neurons and astrocytes, it is primarily localized to astrocytic processes. GAT3 is found near GABAergic synapses, and is believed to participate in the reuptake of presynaptically released GABA (Durkin et al. 1995; Minelli et al. 1996; Ribak et al. 1996a, b; De Biasi et al. 1998).

GAT inhibition as a therapeutic target: The role of GABA and GATs in several disease states will be discussed in the section entitled “GABA and GATs in disease.” The effects associated with GAT inhibition are inherently complicated. A grossly oversimplified view is that GAT inhibition will increase extracellular GABA and therefore be anticonvulsant, antidepressive, anxiolytic, analgesic, and antipsychotic. However, many complicating factors exist. GATs have a variety of functions: (1) termination of inhibitory postsynaptic potentials (IPSPs) (Dingledine and Korn 1985; Roepstorff and Lambert 1994; Draguhn and Heinemann 1996; Engel et al. 1998), (2) supplication of GABA back to axon terminals for reuse (Schousboe et al. 1983), (3) elimination of GABA from diffuse extracellular spaces (Frahm et al. 2001), and (4) nonvesicular release of GABA by reverse transport (Schwartz 1987; Belhage et al. 1993; Gaspary et al. 1998). While re-use and release of GABA enhance inhibition, IPSP termination and reuptake of GABA reduce inhibition. Therefore, the net effect of GAT inhibition is not always foreseeable, and reduced GABA uptake has been shown to have both anti- and proconvulsive effects (During et al. 1995; Patrylo et al. 2001). Tiagabine (TGB), a specific GAT1 inhibitor, may be prescribed as an anticonvulsant, anxiolytic or antidepressant, but it may produce anxiety and depression as side effects in some patients (Kalviainen 2001). Furthermore, it is proconvulsant at high doses and at certain stages of development (Trinka et al. 1999; Balslev et al. 2000). The GAT1 KO mouse has been reported to experience both decreased (Liu et al. 2007) and increased (Chiu et al. 2005) anxiety. The aforementioned conflicting results highlight the complex nature of GABA regulation through the GATs, and

limit the ability to accurately predict experimental outcomes. However, the clinical utility of TGB against a host of neurological disorders supports the use of GAT inhibition as a therapeutic target.

GAT inhibitors: Each GAT transports GABA with a different affinity and possesses a distinct endogenous substrate profile, as shown in Table 1.1. Historically, GABA analogs have been utilized in the search for specific GAT inhibitors. Muscimol is a naturally-occurring GABA analog that interacts with GABA receptors, transporters, and GABA-transaminase (Krogsgaard-Larsen et al. 1975). The realization of the potential of muscimol led to synthesis of analogs with preferential activity towards GABA receptors (THIP (Krogsgaard-Larsen et al. 1977; Krogsgaard-Larsen et al. 2000)) or transporters (THPO, a β-alanine analog (Krogsgaard-Larsen and Johnston 1975; Schousboe et al. 1981)). The synthesis of THPO facilitated the discovery of its cyclic amino acid parents, nipecotic acid and guvacine, which are naturally-occurring GAT inhibitors with no receptor activity (Krogsgaard-Larsen and Johnston 1975). These compounds served as major lead compounds for the development of specific GABA transport inhibitors. While nipecotic acid and guvacine are substrates for GATs, the analogs based off of their chemical structure are not (Clausen et al. 2006). Unfortunately, attempts to discover/synthesize GAT-specific inhibitors that do not have affinity for GAT1 has proven difficult, as discussed further in Chapter 4. The most specific pharmacological inhibitors of the GATs are presented in Table 1.1. A much more detailed reference about the evolution of GABA transport inhibitors is available (Clausen et al. 2006).

Table 1.1: Endogenous GAT substrates and synthesized GAT inhibitors in transfected mouse cells ($IC_{50,}$ μM unless otherwise noted)

	GAT1	BGT1	GAT2	GAT3
GABA	17 [a]	51 [a]	15 [a]	17 [a]
Taurine	- [b]	- [b]	540 [b]	1400 [b]
β-alanine	- [b]	- [b]	28 [b]	99 [b]
Betaine	- [b]	200 [b]	- [b]	- [b]
Tiagabine	0.8	300	>300	800
R,S-EF1502	7	26	>300	>300
R-EF1502	4	22	>150	>150
S-EF1502	120	34	>150	>150
SNAP-5114	>300 [c]	22 [c]	20 [c]	6.6 [c]
NNC-005 2090	19 [c]	1.4 [c]	41 [c]	15 [c]

Table 1: [a]K_m; [b]xenopus oocytes; [c]Rat. References: (Lopez-Corcuera et al. 1992; Liu et al. 1993; Borden 1996; Thomsen et al. 1997; Schousboe and Kanner 2002; Clausen et al. 2005).

While TGB has proven to be valuable clinically, its use is limited by a less than ideal pharmacokinetic profile (i.e., short half-life that mandates dosing three times a day) and side effects (dizziness, fatigue, confusion) (Genton et al. 2001). A better-tolerated GABA uptake inhibitor is therefore desirable. EF1502 (N- [4,4-bis(3-methyl-2-thienyl)-3-butenyl]-4-(methylamino-4,5,6,7-tetrahydrobenzo [d]isoxazol-3-ol) is a compound that has approximately equal potency for GAT1 and BGT1. When EF1502 was co-administered with TGB, a synergistic anticonvulsant effect was observed in the absence of enhanced toxicity (White et al. 2005; Madsen et al. 2009). Furthermore, electrophysiological studies determined that EF1502 decreases spontaneous interictal-like bursting frequency in a hyperexcitable medium, while TGB fails to do so (Smith et al. 2008), demonstrating that the BGT1 action of EF1502 may be functional and may contribute to its anticonvulsant mechanism. The potential ability to target tonic inhibition selectively with BGT1 inhibition due to its presumed extrasynaptic localization suggests that there may be a way to realize the therapeutic effects of BGT1 inhibition without severe disruption of cognitive or behavioral functions, thereby creating a desirable therapeutic profile.

<u>GABA and GATs in disease:</u> GABA and GATs are known to be involved in several diseases as discussed below.

Epilepsy: The GABA hypothesis of epilepsy states that a decrease in GABAergic tone leads to an imbalance between excitatory and inhibitory transmission and increases seizure susceptibility. Pharmacologically increasing GABA leads to decreased seizure activity while decreasing GABA is

proconvulsant (Meldrum et al. 1980). GAT1 is the target of the clinically utilized anticonvulsant TGB and is known to be important in controlling neuronal excitability. Therefore, despite the complex nature of GAT inhibition, the existing clinical data do support the utilization of GAT inhibition as a therapeutic strategy. It has been shown that anticonvulsants working through enhanced inhibition of astrocytic GABA transport are more effective and less toxic than those working preferentially on neuronal GABA transport (White et al. 2002b), supporting the development of agents targeting non-GAT1-mediated transport. In point, the GAT1 over-expressing mouse has increased seizure susceptibility (Ma et al. 2001; Zhao et al. 2003), consistent with decreased available GABA. However, the GAT1 KO mouse was also shown to have slightly increased seizure susceptibility in a small study (Chiu et al. 2005). This result was unexpected, as the KO mouse has massively increased tonic conductance (Jensen et al. 2003; Xu et al. 2007). This result may reflect the inability of the neuron to take up extracellular GABA for subsequent release. Another possible factor is that GAT1 was found to negatively regulate T-cell-mediated immune response in the CNS, and GAT 1 KO mice have an exacerbated inflammatory response post-experimental encephalitis (Wang et al. 2008). Because neuroinflammation contributes to epileptogenesis, GAT-mediated immune system modulation may contribute to the seizure susceptibility of the GAT1 KO mouse.

GAT alterations have been shown to exist in epilepsy and are discussed in the Introduction of Chapter 2. Although GAT1 expression is decreased in epilepsy, TGB retains its ability to increase tonic GABA conductance (Frahm et

al. 2003; Stief et al. 2005). This result supports the following statements: (1) GAT1 is nonetheless functional in epilepsy, (2) GAT inhibition is still a viable target, and (3) GATs are not usually working at full capacity (Dalby 2000; Keros and Hablitz 2005). And so the existing evidence suggests that GATs modify seizures, and seizures alter GATs.

Pain: GABAergic modulation is important in pain as well as epilepsy; in fact, drugs originally marketed as anticonvulsants are becoming increasingly utilized for their antinociceptive potential. Pregabalin, gabapentin, TGB, carbamazepine, and valproic acid are all prescribed for different varieties of pain and possess a mechanism of action involving GABA modulation (Landmark 2007). Of particular interest to this investigation is the observation that TGB, which is an effective treatment for migraine (Landmark 2007), sensory neuropathy (Novak et al. 2001), tonic spasm (Solaro and Tanganelli 2004), bruxism (Kast 2005), and chronic pain (Todorov et al. 2005), exerts its clinical effects through direct and selective inhibition of GAT1. GABA (Castro-Lopes et al. 1993; Ibuki et al. 1997; Eaton et al. 1998) and GABA receptor (Bhisitkul et al. 1990; Castro-Lopes et al. 1995) levels are decreased following axotomy and chronic constriction injury, implicating that a functional loss of GABA in the dorsal horn leads to the development of neuropathic pain in animal models. Activation of both $GABA_A$ and $GABA_B$ receptors in the spinal cord is analgesic (Jasmin et al. 2004). Unfortunately, GABA receptor agonists possess undesirable side effects (Jasmin et al. 2004), warranting the development of GABAergic drugs with different mechanisms of action. Encouragingly, GAT1 inhibition increases GABA levels in

the spinal cord and produces analgesia by reducing the evoked release, not basal levels, of the pain-enhancing excitatory amino acids aspartate and glutamate (Meller et al. 1996; Smith et al. 2007). Furthermore, a decrease in GAT1 expression results in hypoalgesia (Xu et al. 2008) while upregulation is hyperalgesic (Ng and Ong 2001; Hu et al. 2003). GAT1 is upregulated following chronic constriction injury and contributes to pain behavior (Daemen et al. 2008). As the ability of GAT1 inhibition to decrease pain is attributed to a direct increase in GABA levels, it is possible that non-GAT1 inhibitors will possess antinociceptive or analgesic actions as well. GAT1 inhibition is relatively well tolerated; however, side effects are observed. Non-GAT1 (i.e., BGT1) inhibition may potentially incur a decreased incidence of side effects due to its supposed effect on tonic inhibition rather than phasic neurotransmission.

Depression: GABAergic neurotransmission is also recognized to be an important modulator in depression. Patients with depression have lower levels of plasma, cerebrospinal fluid, and brain GABA , as well as a decreased density of GABAergic neurons in the orbitofrontal cortex (Rajkowska et al. 1999; Sanacora et al. 1999, 2000; Krystal et al. 2002). Preclinical data reveal that animals that have been exposed to a depressive stimulus (a learned helplessness paradigm) have a reduced release of GABA and a decreased influx of chloride in several brain areas (Petty and Sherman 1981; Borsini et al. 1988; Drugan et al. 1989; Gomez et al. 2003; Briones-Aranda et al. 2005). In agreement with these data, GABA release and chloride uptake are increased following pharmacological antidepressant treatment (Petty and Sherman 1981; Malatynska et al. 1995;

Kram et al. 2000; Gomez et al. 2003; Herman et al. 2003; Yang and Shen 2005). Clinical studies have provided some evidence of efficacy for several GABAergic drugs for the treatment of depression, including valproic acid, vigabatrin, gabapentin, and TGB (Kalueff and Nutt 2007). The modulation of depression by GAT1 is demonstrated not only by the efficacy of TGB, but also by the reduced susceptibility to a learned helplessness paradigm of GAT1 KO mice compared to WT controls (Liu et al. 2007). The effect of non-GAT1 inhibition on depression has not been determined.

Anxiety: Anxiety is also associated with GABAergic dysfunction (Nutt and Malizia 2001; Lydiard 2003; Nemeroff 2003). In humans and animal models, GABA receptor activation is anxiolytic while antagonism produces anxiety (Kalueff and Nutt 1996). In complement to this, valproic acid, vigabatrin, and TGB are clinically effective anxiolytics (Lang and de Angelis 2003; Nemeroff 2003; Rosenthal 2003; Stahl 2004). The modulation of anxiety by GAT1 is supported by the anxiolytic efficacy of TGB and also by the GAT1 KO mouse, which has been reported to display decreased anxiety compared to WT mice (Liu et al. 2007).

Neuropsychiatric disorders: Several neuropsychiatric disorders involve dysfunction of the GABAergic system. Of particular relevance to this proposal are diseases involving a manic phase, such as bipolar disorder and schizophrenia. Schizophrenia has been associated with decreased interneuron and GABAergic terminal numbers, decreased GABA receptor binding affinity, and altered input into GABAergic cells (Benes and Berretta 2001). The GABA hypothesis of schizophrenia suggests that insufficient GABA-mediated inhibition of

dopaminergic neurons is responsible for the disorder (Koran 1976; Wolkowitz and Pickar 1991). Selective loss of nonpyramidal neurons in CA2 of the hippocampus has been described in schizophrenia and bipolar disorder (Benes et al. 1998; Todtenkopf et al. 2005). Although it is accepted that there is a decrease in GABAergic functioning in various neuropsychiatric disorders, the responsible mechanisms are fundamentally different, and clear endophenotypes involving the GABA system exist for each disorder (Benes 2007). The involvement of the GABA system in neuropsychological disorders is highly complex, but it remains evident that GABA modulation is a rational strategy for the treatment of these disorders. GABA-modulating drugs are clinically efficacious against schizophrenia (Wassef et al. 1999) and bipolar disorder (Bowden 1998). TGB has been determined inappropriate for the treatment of acute mania (Carta et al. 2002). Beyond this, however, there have been no adequate clinical studies regarding the use of TGB for neuropsychiatric disorders. One small randomized, double-blind, placebo-controlled study and a limited number of case studies suggest possible efficacy (Kaufman 1998; Vieta et al. 2006); nonetheless, it is important to note that any firm conclusion regarding the efficacy of TGB in neuropsychiatric disorders will necessarily have to be provided by appropriate double-blinded, placebo-controlled clinical trials. The utility of non-GAT1 inhibitors has not been determined for any of these disorders.

As can be readily deduced from the above discussion, GAT inhibition has the potential to be useful in the treatment of several diverse neurological disorders. While GAT1 inhibition has been shown to be clinically valuable in several

disorders, it remains to be determined if inhibition of the other GATs, including BGT1, would provide a therapeutic benefit as well.

Osmoregulation and osmolyte transporters

Functions of betaine: Betaine is found ubiquitously in nature in animals, plants, and microorganisms. Betaine serves two main functions: to serve as an organic osmolyte and as a source of methyl groups. Betaine is accumulated in higher organisms mostly through the diet. Wheat, spinach, seafood, and beets contain high amounts of betaine, and the average daily intake for humans is 1 g/day. Although 9-15 g/day has been reported to be safe, 3-6 g/day for more than 4 weeks has produced cerebral edema in children (Yaghmai et al. 2002; Devlin et al. 2004). Betaine is absorbed rapidly (1-2 h) into the body through BGT1 and amino acid transport system A and resting serum concentrations are 20-70 μM (Lever et al. 1994; Peters-Regehr et al. 1999; Schwahn et al. 2003). In rats, plasma concentrations are approximately 190 μM, while the brain concentration is near 50 μM (Slow et al. 2009). Both BGT1 and amino acid transport system A also are present at the BBB (Smith 2000; Takanaga et al. 2001). Betaine is also formed in the body, predominantly in the liver and kidney, by the oxidation of choline. Choline is initially converted to betaine aldehyde by choline dehydrogenase in mitochondria. Betaine aldehyde is then converted into betaine in the mitochondria and cytosol via oxidation by betaine aldehyde dehydrogenase.

Betaine is a zwitterionic quaternary ammonium (molecular weight 117.2) and is a derivative of the amino acid glycine. It belongs to the family of methylamines due to its three chemically reactive methyl groups. These groups allow betaine to function as a methyl donor in transmethylation reactions that are important in many biochemical pathways. This catabolic transmethylation reaction of betaine is catalyzed by betaine homocysteine methyl transferase to form dimethylglycine. Betaine that is not catabolized is available to function as an organic osmolyte.

Many aspects of cellular function are directly affected by osmotic state. These functions include protein turnover, pH control, membrane transport systems, gene expression, and amino acid, fatty acid, carbohydrate, and ammonia metabolism (Haussinger 1996). Osmotic balance is maintained in part by organic osmolytes. The initial adaptation process to osmotic stress involves the accumulation of low molecular weight inorganic ions such as K^+, Na^+, and Cl^-. While ameliorating the osmotic perturbation, these ions have a destabilizing effect on intracellular protein structure and enzymatic function. Therefore, cells replace the inorganic ions with organic osmolytes (Petronini et al. 1993; Burg 1994). Organic osmolytes may also function as a 'chemical chaperone' for protein folding in a process known as osmotic remediation (Burg 1995). Important organic osmolytes in the CNS include taurine, myo-inositol, sorbitol, and betaine. The ability of cells to accumulate these depends on the expression of the transporter or biosynthetic enzyme specific for each osmolyte: the taurine transporter (TauT) for taurine, the sodium-myoinositol cotransporter (SMIT) for

myo-inositol, and BGT1 for betaine. Sorbitol is synthesized intracellularly by the enzyme aldose reductase (AR).

Sodium, potassium, chloride, bicarbonate, glucose and urea are the osmotically important body fluid solutes. Resting serum osmolality is approximately 285-295 mOsm. Serum osmolality above 380 mOsm produces stupor, 400 mOsm leads to generalized tonic clonic seizures, and 420 mOsm is fatal (Habel 1999).

Osmoregulation in epilepsy: BGT1 not only transports GABA, but also betaine (Lopez-Corcuera et al. 1992). Network excitability is directly affected by the size of the extracellular space (ECS) (Roper et al. 1992), which is determined by cellular size. Osmolytes, such as betaine, accumulate in cells and cause water retention to avoid cell shrinkage in hyperosmotic solutions (Yancey et al. 1982). Excessive neuronal activity causes a hypertonic extracellular environment, cell swelling, and ECS reduction (Dietzel et al. 1982). ECS reduction magnifies the effects of extracellular molecules because (1) there is less volume of dilution, (2) diffusion is slowed due to less space and more tortuosity, and (3) electrical conduction is enhanced by increased resistance (Schwartzkroin et al. 1998). Accordingly, a reduced ECS is proconvulsant, while an enlarged ECS suppresses seizures (Dudek et al. 1990). Since BGT1 can regulate the osmotic state of the CNS, it may be important in controlling neuronal excitability.

Osmolyte transporters in epilepsy: The involvement of osmolyte transporters in epilepsy is less clear than that of GATs; however, several associations exist and are described in the Introduction of Chapter 2.

The Betaine/GABA transporter

Regulation: BGT1 is encoded by the SLC6a12 gene. Eight isoforms exist, which differ only in their 5' untranslated regions. By this 5' end motif, BGT1 is grouped into three types; A, B, and C, which are all hypertonicity-inducible in MDCK cells and display tissue-specific expression. Type A is expressed in the kidney medulla, Type B in the kidney medulla and cortex, brain, and liver, and Type C in the kidney medulla and cortex and the brain. The physiological significance of these different isoforms is unknown (Takenaka et al. 1995). The regulatory mechanisms of SLC6a12 and BGT1 protein expression in the CNS have not been elucidated. However, many studies have addressed these mechanisms in cell systems outside the CNS, and are the basis for the following discussion. Expression of BGT1 is altered by tonicity due to its promotion by the transcription factor tonicity-responsive enhancer binding protein (TonEBP), also known as osmotic response element-binding protein (OREBP) and nuclear factor of activated T-cells 5 (NFAT5). The N-terminus of TonEBP contains its DNA binding domain (Stroud et al. 2002), while the C-terminus contains an NaCl dependent transactivating domain (Ferraris et al. 2002b). TonEBP binds to tonicity-responsive enhancers on the BGT1 gene, TonE1 and TonE2, which act synergistically to promote expression (Takenaka et al. 1994; Miyakawa et al.

1998; Miyakawa et al. 1999a). Synergism suggests that a protein-protein interaction that stabilizes DNA binding exists between two TonEBP molecules bound to TonE1 and TonE2. Such a complex is commonly seen (Tanaka 1996). Under osmotic stress, TonEBP expression increases, and it is translocated to the nucleus (Woo et al. 2000a) to promote the osmoprotective genes BGT1, TauT, SMIT, and AR. Expression of BGT1 may be increased by proteins that contribute to the activation of TonEBP, including activator protein-1 (Irarrazabal et al. 2008), protein kinase A (Ferraris et al. 2002a), phosphatidylinositol 3-kinase 1A (Irarrazabal et al. 2006), heat shock protein 90 (Chen et al. 2007), p38, and Fyn (Ko et al. 2002). In contrast, RNA helicase A (Colla et al. 2006) and poly(ADP-ribose) polymerase-1 (Chen et al. 2007) produce an inhibitory action on TonEBP transactivating activity. All of the aforementioned targets contribute to the regulation of TonEBP, but none is completely necessary. There is extensive post-transcriptional control over the surface expression of BGT1. Sorting signals and a PDZ association motif are found at the intracellular C-terminus of BGT1 (Perego et al. 1999). Extracellular calcium can cause internalization of BGT1, which may be PKC mediated (Kempson et al. 2006). PKC-mediated phosphorylation disrupts the association of BGT1 with the PDZ domain of Lin7 (Massari et al. 2005), an interaction that is necessary for BGT1 to remain at the membrane, but not for it to be targeted to the membrane (Perego et al. 1999). PDZ domains anchor transmembrane proteins to the cytoskeleton and tether signaling complexes together, and Lin7 is a synaptic protein important in synaptic complex assembly. De novo synthesis and microtubule-dependent membrane

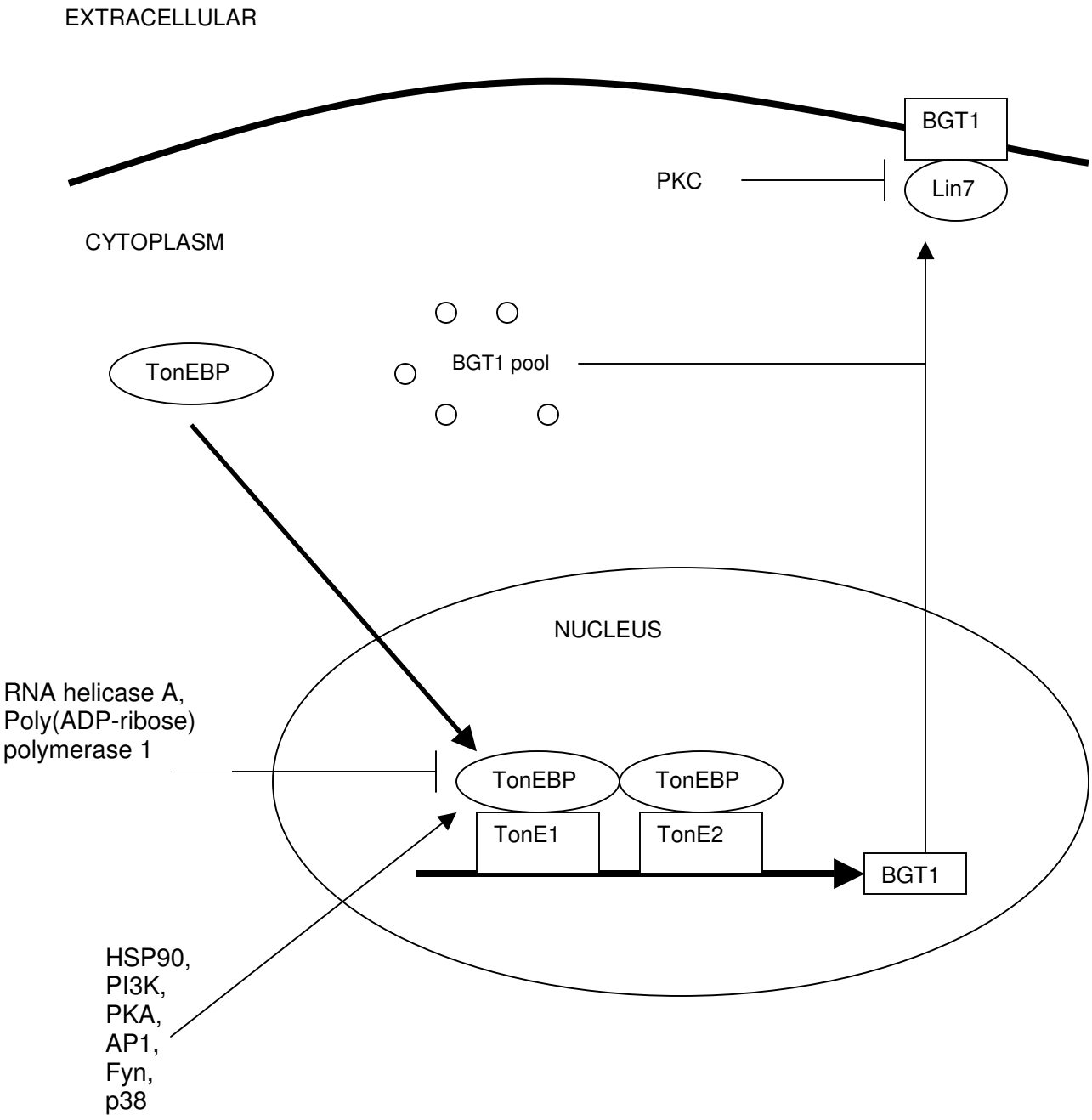
trafficking of cytosolic BGT1 pools were shown to contribute to hypertonicity-induced BGT1 upregulation (Basham et al. 2001; Kempson et al. 2003). A summary of the proposed regulation of BGT1 is shown in Figure 1.1. The regulation of the osmoprotective genes in the brain is investigated and discussed in Chapter 2.

Functions in the CNS: It has been suggested that BGT1 may not function to remove presynaptically released GABA due to its expression levels, localization, and affinity. However, several potential functions for BGT1 in the CNS remain, including: 1) osmotic regulation, 2) GABA and/or betaine transport across the blood-brain barrier, 3) redistribution of GABA from GABAergic to glutamatergic neurons, 4) redistribution of betaine following osmotic stress, 5) functionality as a neurotransmitter-gated ion channel. These possible roles for BGT1 are described and supported further in the Discussion of Chapter 5.

Mouse models

Several mouse models were utilized for the studies described in this dissertation. Each model has been validated for the intended purpose in the literature, and was validated for use in this laboratory prior to experimental testing included in this work. A very brief introduction to each model is presented here. Methods are presented in the chapters that utilize the tests.

Figure 1.1: The proposed regulation of BGT1 expression in the kidney. TonEBP is translocated to the nucleus where it binds to TonE promoter sites to induce the transcription of BGT1. Several effectors contribute to the transactivating activity of TonEBP: heat shock protein 90 (HSP90), phosphatidylinositol 3-kinase 1A (PI3K), protein kinase A (PKA), activating-protein family 1 (AP-1), Fyn, p38, RNA helicase A, and poly(ADP-ribose) polymerase 1. BGT1 is maintained at the membrane by interaction with Lin7, which is disrupted by protein kinase C (PKC)-mediated phosphorylation.


EXTRACELLULAR
BGT1
PKC
Lin7
CYTOPLASM
TonEBP
BGT1 pool
NUCLEUS
RNA helicase A,
Poly(ADP-ribose)
polymerase 1
TonEBP
TonEBP
TonE1
TonE2
BGT1
HSP90,
PI3K,
PKA,
AP1,
Fyn,
p38

Minimal clonic threshold: The minimal clonic seizure threshold is a measure of forebrain seizure threshold. These seizures experimentally present as jaw and forelimb clonus and may involve rearing and falling (White et al. 2002a).

6 Hz psychomotor seizure test/threshold: The 6 Hz test is a model of complex partial seizures involving limbic structures. The experimental 6 Hz seizure presents as a clonic phase followed by stereotypic, automatistic behaviors such as jaw chomping and vibrissae twitching. This test is an acute seizure test that displays pharmacoresistance to several of the clinically available AEDs including phenytoin, carbamazepine, lamotrigine, and topiramate (White et al. 2002a).

Maximal electroshock test/minimal tonic extension threshold: The MES test represents hindbrain seizures and experimentally manifests as a tonic-extension seizure. Drugs that display efficacy in this test are clinically efficacious against tonic-clonic seizures (White et al. 2002a).

Corneal kindling: Corneal kindling produces partial seizures that secondarily generalize. This model represents a cost-effective alternative to the highly clinically predictive but labor-intensive electrically kindled rat. The validation of the corneal kindled mouse model as a pharmacological tool is discussed in detail in Chapter 3.

i.v. pentylenetetrazol (i.v.PTZ) threshold: The i.v.PTZ test is an acute chemical test that is used to determine what effect a drug or genetic manipulation has on seizure threshold. Seizures are induced by the infusion of PTZ, a GABA receptor antagonist. The occurrence of a seizure does not have to be abolished; the measure taken is the dose of PTZ required to produce the first twitch and full

clonus in a population of animals. Hence, it is a highly sensitive parametric method for seizure threshold assessment (Orlof 1949).

Frings mouse: The Frings mouse is a model of sensory evoked reflex epilepsy, which usually manifests as primary generalized tonic-clonic seizures. These mice experience tonic-extension seizures in response to an audiogenic stimulus due to a mutation in the mass1 protein (Skradski et al. 1998). The Frings mouse is advantageous to the DBA/2 mouse model of audiogenic seizures because DBA/2 mice can only be utilized at a young age (2-4 weeks) due to deafness, and also have a high mortality rate following seizure activity (Le Gal La Salle and Naquet 1990).

Pilocarpine-induced status epilepticus: Status epilepticus (SE) is characterized by the occurrence of unremitting seizure activity, usually longer than 30 min. It can be experimentally induced by administration of a muscarinic agonist, pilocarpine. Pilocarpine-induced SE (pilo-SE) leads to pathological changes similar to those observed in humans with temporal lobe epilepsy, i.e., mossy fiber sprouting, astrogliosis and hippocampal sclerosis, characteristic cell loss in the CA1 of the hippocampus, and the development of spontaneous seizure activity following a postinsult latent period (2-4 weeks in the mouse pilo-SE model) (Cavalheiro et al. 1996; Shibley and Smith 2002).

Forced swim test: The forced swim test involves a preconditioning of animals, whereby they are placed in a cylinder of water from which they cannot escape prior to the recorded portion of the test. This preconditioning induces “depression,” or “learned helplessness.” Animals that are more susceptible to this

learned helplessness will give up more quickly than normal animals, and animals with antidepressant treatment will swim for a longer period of time (Porsolt et al. 1977).

Writhing test: The writhing test is a model of acute somato-visceral pain induced by an intraperitoneal injection of dilute acetic acid. A ‘writhe’ is caused by a wave of abdominal wall constrictions and elongations, usually accompanied by a hindlimb extension. The writhing test is not an inflammatory-mediated process, although inflammation does occur (Northover 1963; Collier et al. 1968). This test is sensitive to broad range of analgesics, making it an attractive model for the initial identification of analgesic agents. However, it is criticized for determining analgesic potential in compounds that are nonanalgesic and because of the existence of a large number of nonresponders (Siegmund et al. 1957; Hendershot and Forsaith 1959; Collier et al. 1968; Pearl et al. 1969).

Carrageenan test: Carrageenan is a linear polysaccharide extracted from seaweed that, upon subcutaneous injection, causes hyperalgesia and localized inflammation (Hargreaves et al. 1988). Hyperalgesia develops relatively slowly in the carrageenan test (h) and involves a phenomenon termed “windup,” in which the number of neuronal responses progressively increases (Mendell 1966). Windup is involved in the maintenance of inflammatory and neuropathic pain and is attributed to responses of spinal dorsal horn neurons evoked by C-fiber afterdischarge (Dubner 1986; Gracely et al. 1992). A specific receptor has not been identified in the carrageenan-induced inflammatory pain response.

Formalin test: The formalin test was originally designed for use in rats (Dubuisson and Dennis 1977) and later modified for use in mice (Takahashi et al. 1984). The formalin test has become one of the most commonly used assays for chemically induced nociception (Mogil et al. 2001). Formalin injection into the plantar region of a hind paw elicits a characteristic biphasic licking of the affected paw: the mouse licks its paw for 5-10 min immediately following the injection (acute phase or phase 1), followed by a brief latent period where there is little activity (usually less than 5 min), followed by a 20-30 min period of licking (inflammatory phase or phase 2). The acute phase is believed to be mediated by direct nociceptor activation, while the inflammatory phase is attributed predominantly to ongoing peripheral afferent input and controversially to central sensitization (Dallel et al. 1995; Taylor et al. 1995).

Tail flick: The tail flick test measures thermal sensitivity utilizing the flexor withdrawal reflex. This reflexive behavior in response to thermal pain is predictive of compounds with analgesic efficacy in animals (D'Amour and Smith 1941; Taber 1973) and humans (Chan and Dallaire 1989).

Locomotion: Increased locomotor activity in mice is a model of the manic phase of neuropsychiatric disorders in humans, which can be induced by stimulation of the dopaminergic system (Mamelak 1978; Peet and Peters 1995; Anand et al. 2000). In experimental and certain human cases, lithium attenuates this effect (Van Kammen and Murphy 1975; Berggren et al. 1978; Huey et al. 1981; van Kammen et al. 1985). Mice with increased basal locomotion may

possess inherently altered neurobiology compared to normal rodents, and may serve as a genetic model of bipolar disorder or schizophrenia.

Hypothesis and dissertation overview

The goal of this dissertation was to test the hypothesis that BGT1 expression plays a role in epilepsy, seizure susceptibility, and other pathological states. Chapter 2 explores the alterations in BGT1 mRNA expression following status epilepticus compared to other GABA and osmolyte transporters, as well as possible mechanisms regulating the brain expression of BGT1 mRNA. The results from these studies suggested that BGT1 expression is involved in epilepsy and warranted further investigation into its role in controlling seizure activity and possibly other behaviors modulated by GABA. A two-pronged approach was utilized for this determination: pharmacological inhibition of BGT1 and genetic knockout of BGT1. Chapter 3 validates the use of the corneal kindling model for pharmacological testing, and Chapter 4 utilizes this test, along with several others, to determine if pharmacological inhibition of BGT1 has an effect on seizure activity and other GABA-modulated behaviors including pain and depression. During the course of investigation, the pharmacological inhibitor utilized in Chapter 4 was determined to be nonspecific for BGT1. Therefore, the BGT1 KO mouse became the only available tool for accurately testing BGT1's involvement in seizure control and behavior. Data obtained in this investigation are presented in Chapter 5. The studies performed in this dissertation illustrate that BGT1 appears to be involved in epilepsy and behaviors modulated by GABA

such as depression, anxiety, locomotion, and pain; however, as evidenced by extensive seizure threshold studies, BGT1 does not appear to be directly involved in controlling seizure threshold. Nonetheless, the findings described herein support the further evaluation of BGT1 as a potential therapeutic target for a number of neurological disorders including depression, anxiety, and other neuropsychiatric disorders involving a manic phase.

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CHAPTER 2

HIPPOCAMPAL BETAINE/GABA TRANSPORTER mRNA EXPRESSION IS NOT REGULATED BY INFLAMMATION OR DEHYDRATION POSTSTATUS EPILEPTICUS

Introduction

GABA is the primary inhibitory neurotransmitter in the mammalian CNS, and after its release from interneurons, GABAergic signaling is terminated via reuptake by GABA transporters (Madsen et al. 2010). The betaine/GABA transporter (BGT1) is able to transport the organic osmolyte betaine as well as GABA, and is therefore both an osmolyte transporter and a GABA transporter (GAT). A variety of GAT alterations have been shown to exist in epilepsy. GAT1 and GAT3 expression are altered in a time-dependent manner following seizure activity; GAT1 may transiently increase postseizure but is consistently downregulated chronically. Similarly, GAT3 has been reported to both increase and to decrease following seizure activity (Hirao et al. 1998; Ueda and Willmore 2000; Andre et al. 2001; Patrylo et al. 2001; Sperk et al. 2003; Zhu and Ong 2004a). GABA transporters display altered distribution and decreased function in tissue from humans with epilepsy (During et al. 1995; Mathern et al. 1999; Patrylo et al. 2001). In the rat kainic acid model of status epilepticus (SE), BGT1

expression was reported to increase post-SE (Zhu and Ong 2004a). GAT downregulation in epilepsy may serve to increase tonic inhibitory conductance by increasing the extracellular GABA concentration. However, these transporters are able to reverse and release GABA under certain conditions such as elevated extracellular K^+ and glutamate (Gaspary et al. 1998; Heja et al. 2009), which is known to occur during seizures (Krnjevic et al. 1982; Somjen and Giacchino 1985; Liu et al. 1997). Downregulation of these transporters, then, may exacerbate seizure activity because transporter reversal may be important in seizure cessation or in preventing seizure spread (Patrylo et al. 2001). Indeed, it has been shown that glutamate causes a significant increase in extracellular GABA in nonepileptogenic hippocampi, but not in epileptogenic hippocampi from patients with refractory epilepsy (During et al. 1995). However, GAT inhibition remains a viable anticonvulsant strategy despite seizure-related downregulation as demonstrated by the clinical efficacy of the specific GAT1 inhibitor, tiagabine (Madsen et al. 2010). The role of BGT1 in controlling neuronal excitability is unknown. However, it is evident that inhibition of GAT1 by tiagabine can control seizures and that seizures can alter the expression of GAT1 and GAT3.

The role of osmolyte transporters in epilepsy is less clear, but several associations exist. Organic osmolytes accumulate in cells and cause water retention to avoid cell shrinkage in hyperosmotic solutions without disturbing cellular function (Yancey et al. 1982; Lang et al. 1998; Wehner et al. 2003; Strange 2004). Dehydration causes the upregulation of genes responsible for the synthesis of osmolyte transporters or biosynthetic enzymes of organic osmolytes

(Burg et al. 1997; Waldegger and Lang 1998; Handler and Kwon 2001). Through this mechanism, osmolyte transporters modulate the size of the extracellular space (ECS) by exerting control over cell size. Excessive neuronal activity, such as that associated with epileptiform bursts, causes cell swelling and ECS reduction (Dietzel et al. 1982). A reduced ECS enhances neuronal excitability, while an increased ECS dampens excitability (Dudek et al. 1990; Schwartzkroin et al. 1998). Therefore, osmotic homeostasis is important in controlling neuronal excitability. In addition, seizure-induced alterations to osmotic stress have been described. The Na^+/myo-inositol co-transporter (SMIT) is significantly increased post-SE (Nonaka et al. 1999). Furthermore, levels of the organic osmolyte, taurine, are altered by seizures (Lehmann et al. 1985; Baran 2006), and BGT1 expression is reportedly increased following kainic acid-induced SE (Zhu and Ong 2004a). In addition, betaine, myo-inositol and taurine all display anticonvulsant activity, suggesting a direct interaction between osmotic status and seizure susceptibility (Freed et al. 1979; French et al. 1986; Patishi et al. 1996; Solomonia et al. 2007; Junyent et al. 2009).

Transcription plays an essential role in the upregulation and insertion of BGT1 into the cell membrane. Increased transcription, rather than increased mRNA stability, is responsible for a significant proportion of the hypertonicity-induced increase in BGT1 (Uchida et al. 1993; Lammers et al. 2005) and aldose reductase (AR) (Garcia-Perez 1995). There is also extensive post-transcriptional control over the surface expression of BGT1. De novo synthesis and membrane trafficking of cytosolic pools were shown to contribute to hypertonicity-induced

BGT1 upregulation in the kidney (Basham et al. 2001; Kempson et al. 2003). In the absence of a specific antibody that can reliably detect BGT1 in brain tissue, mRNA studies remain able to produce meaningful results since de novo synthesis is known to contribute to BGT1 upregulation in other tissues.

The known transcription factor for BGT1 is the tonicity-responsive enhancer binding protein (TonEBP). TonEBP expression increases and it is translocated to the nucleus in response to dehydration in the kidney (Woo et al. 2000b) to promote the expression of osmoprotective genes: the osmolyte transporters BGT1, the taurine transporter (TauT), and SMIT, as well as the intracellular enzyme responsible for the production of the osmolyte sorbitol, aldose reductase (AR) (for review see (Woo et al. 2002)). The osmoprotective genes are able to increase in the brain in response to hypertonicity and cause alterations in osmolyte tissue content (Heilig et al. 1989; Lien et al. 1990; Verbalis and Gullans 1991; Ibsen and Strange 1996; Minami et al. 1996; Bitoun and Tappaz 2000b). While TonEBP is believed to be the major transcriptional regulator of the osmoprotective genes in the kidney and is able to perform bidirectional regulation of the osmoprotective genes (Burg et al. 1996; Miyakawa et al. 1999b; Woo et al. 2000a; Na et al. 2003; Lopez-Rodriguez et al. 2004), this does not appear to be true in the brain. For example, TonEBP is expressed differentially from its downstream osmoprotective genes in the brain with regard to cell type, region, and expression level (Maallem et al. 2006b). Furthermore, the expression of TonEBP and of each osmoprotective gene varies by tissue (Zhang et al. 2003). Finally, TonEBP is only appreciably present and hypertonic-inducible in neurons

in the brain (Loyher et al. 2004; Maallem et al. 2006a), yet other non-neuronal cells, such as astrocytes and endothelial cells, can upregulate the osmoprotective genes in response to osmotic stress, suggesting a TonEBP-independent mechanism (Isaacks et al. 1994; Strange et al. 1994; Wiese et al. 1996; Bitoun and Tappaz 2000a; Petronini et al. 2000; Maallem et al. 2006b).

Recently, it has been suggested that an endogenous inhibitory GABAergic system exists within the immune system, which includes the presence of GATs on T cells and macrophages (Bhat et al. 2010). Furthermore, inflammation has been shown to modulate the expression of GATs. GAT1 and GAT3 are increased in the spinal trigeminal nucleus following carrageenan injection, a response likely to contribute to hyperalgesia (Ng and Ong 2001). Following an encephalitic insult, GAT1 is significantly downregulated in the spinal cord of WT mice, and the GAT1 KO mouse has an exacerbated response to an encephalitic insult, suggesting that GAT1 is an important modulator of antigen-specific T cell responses (Wang et al. 2008). TonEBP is also known as nuclear factor of activated T-cells 5 (NFAT5), an inflammatory-mediated transcription factor. Despite a significant increase in TonEBP in response to an inflammatory stimulus in T lymphocytes, AR did not increase (Trama et al. 2000). Furthermore, immunosuppressant drugs do not alter the hypertonicity-induced upregulation of BGT1, and different immunosuppressants display divergent effects regarding SMIT activity (Atta et al. 1999). The available data indicate that 1) TonEBP may regulate the osmoprotective genes only in response to osmotic stimuli, not to inflammatory stimuli, and 2) TonEBP regulation of osmoprotective genes is only

relevant in select tissues, but not in the brain. As discussed above, this expression pattern was compared to that of other GABA transporters as well as osmoprotective genes.

The current study was performed to determine if BGT1 mRNA (SLC6a12) expression is altered in the hippocampus following SE, and to investigate whether inflammation and/or dehydration may contribute to the BGT1 response pattern in the brain.

Methods

Animals: Adult male C57/B6 mice weighing a minimum of 18 grams (Charles River, Kingston, WA) were used. Animals were housed in a temperature-, humidity-, and light-controlled (12 h light: dark cycle) facility. Mice were group-housed and permitted free access to food and water except during the dehydration protocol. All experimental procedures were performed in accordance with the guidelines established by the National Institutes of Health (NIH) and received approval from the University of Utah's Animal Care and Use Committee (IACUC).

Pilocarpine-induced status epilepticus (SE): Pilocarpine SE was induced by injection of 330 mg/kg (i.p.) pilocarpine hydrochloride in 0.9% saline (Mazarati et al. 2004) (Sigma, St. Louis, MO). -/- scopolamine methyl-bromide (1 mg/kg, Sigma-Aldrich, St. Louis, MO) was administered 30 min prior to pilocarpine injection to reduce peripheral cholinergic effects (Shibley and Smith 2002). Mice were observed for SE; animals displaying at least three stage 3-5 seizures

according to a modified Racine scale (Racine 1972), or at least one stage 5 seizure accompanied by continuous clonus were considered to have undergone SE. The motor component of SE was abolished after 60 min by an injection of diazepam (10 mg/kg, i.p., Sigma-Aldrich, St. Louis, MO). Mice in the control group received 0.9% saline in place of pilocarpine; otherwise they were treated identically to experimental animals.

Induction of inflammation and dehydration: mRNA levels of SE animals were compared to levels from animals 24 h following LPS injection (4 mg/kg, i.p., strain 0111:B4, Sigma-Aldrich, St. Louis, MO) or after 24 h of water withdrawal (n=10 per group). The presence of an inflammatory response was confirmed utilizing core body temperature and weight measurements. Body temperatures were determined at baseline and hly for 4 h postinjection, then again at 8 h and 24 h. The peak body temperature reached by each mouse was compared to its individual baseline temperature, and the temperature change was determined for each mouse in order to normalize for mice with low or high basal temperature. Dehydration was determined by plasma osmolality values. Blood was obtained from mice by decapitation and centrifuged for 3 min at >3,000 g to separate plasma. Plasma osmolality was determined utilizing an osmometer (Advanced Instruments, Inc., Model 3320 Osmometer, Norwood, MA).

RNA extraction and cDNA synthesis: Eight, 24, and 72 h, 1 week and 4 weeks post-SE, animals were sacrificed by cervical dislocation and hippocampi were dissected out for gene expression determination (n=6-9 per group). RNA was extracted and processed with RNeasy miniprep kits (QIAGEN, Valencia, CA)

according to the manufacturer's instructions. A NanoDrop 3300 Spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA) was utilized to calculate RNA concentrations based on spectral absorbance at 260/280 nm. A standard RT reaction was run to produce cDNA. Two µg of total RNA, 1 µl of 250 ng/µl random hexamers, 1 µl of 10 mM dNTPs, and water to a volume of 13 µl were heated to 65 ℃ for 5 min. Four µl of 5x First Strand buffer, 2 µl of 0.1 M DTT, and 1 µl of SuperScript II (Invitrogen, Carlsbad, CA) were added and the reaction run at 42 ℃ for 50 min then 70 ℃ for 15 min. Fifty µl of purified water was added and samples stored at -20℃ until use.

Quantitative PCR: Osmolyte-related, GAT1 and GAT3 internal standards for PCR were cloned, while the standards and primers for BGT1 were purchased from Origene (Rockville, MD). The University of Utah DNA Sequencing Core Facility confirmed the cloned DNA sequences and a BLAST search was performed to confirm gene specificity. Primer sequences and product sizes are presented in Table 2.1. The quantitative PCR reaction contained 12.5 µl of RT^2 Real-Time SYBR Green PCR Master Mix (SA Biosciences, Frederick, MD), 10.5 µl of purified water, 1 µl of 10 µM primer set, and 1 µl of cDNA template. Quantitative PCR was performed using a PTC-200 Peltier Thermal Cycler with a Chromo4 Continuous Fluorescence Detector (MJ Research Inc., Waltham, MA). Data was analyzed with MJ Opticon Monitor Analysis Software, Version 3.00 (Bio-Rad Laboratories, Hercules, CA). All values were normalized to Proteosome subunit β (Psmb6), a housekeeping gene with minimal variability (Rubie et al.

2005). Primer sequences, annealing temperatures, and product sizes are presented in Table 2.1. Each gene was amplified for 35 cycles.

Analysis and statistics: All genes were normalized to the value of the housekeeping gene, Psmb6, for that sample and expressed relative to a mean control value of 100. Results are presented as mean ± SEM. A two-tail Student's t-test was utilized prior to normalization to determine significant differences between control and experimental groups. Differences were considered significant at $p < 0.05$.

Results

The mRNA expression levels of BGT1, GAT1, GAT3, TauT, SMIT, AR, and TonEBP were determined 8 h, 24h, 72h and 1 and 4 weeks following pilocarpine-induced SE. Figure 2.1 displays the temporal expression pattern of the genes following SE. Eight h following SE, SMIT mRNA was increased significantly, while GAT1 mRNA was decreased compared to control values. At 24 h post-SE, BGT1 mRNA expression was significantly increased, as were the osmolyte transporters SMIT and TauT. GAT3 mRNA was decreased whereas GAT1 mRNA had returned to control values. At the 72 h time-point BGT1 mRNA expression was significantly decreased along with GAT1 and GAT3, while SMIT remained increased. Hence, the expression of BGT1 switched from being similar to that of the osmolyte transporters early on post-SE (24 h) to being more like that of the GABA transporters at a later time-point (72 h). This pattern was again observed 4 weeks post-SE, when BGT1, GAT1, and GAT3 mRNA were downregulated, while SMIT was upregulated. AR mRNA expression was not

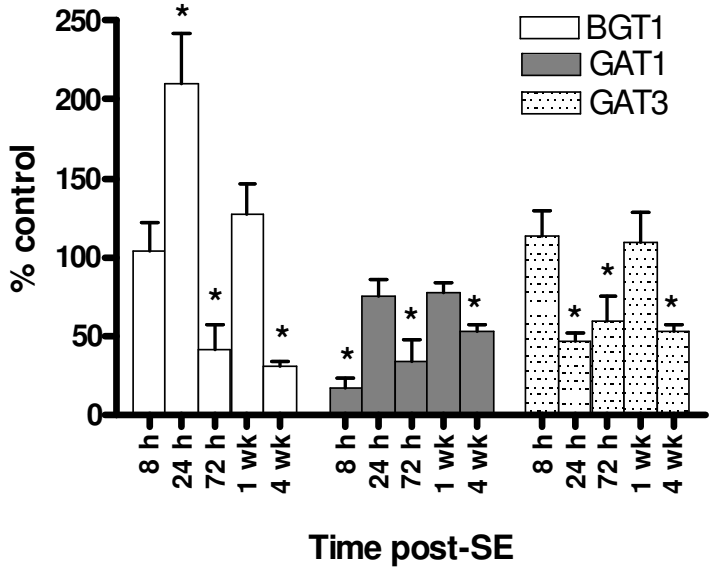
Table 2.1: Primers for PCR amplification

Gene	Forward Primer	Reverse Primer	AT	PS (bp)
GAT1	taacaacaacagcccatcca	ggagtaaccctgctccatga	60℃	326
GAT3	tttggtcttccccttttcct	aagactccactcaaccccct	60℃	214
SMIT	cactctgagtggatacttcc	tctcttaacttcctcaaacc	52℃	544
TauT	tccacaaagacatcctgaagc	ggtgaagttggcagtgctaag	60℃	539
AR	ttgactgcgcccaggtgtac	tatatgctgtcaccacgatgc	60℃	504
TonEBP	atgcaatttcagaatcagcc	gcatttgctgagaaagaag	60℃	514
Psmb6	tctgatggcaggaatcat	acatagccatagatgtacga	57℃	137

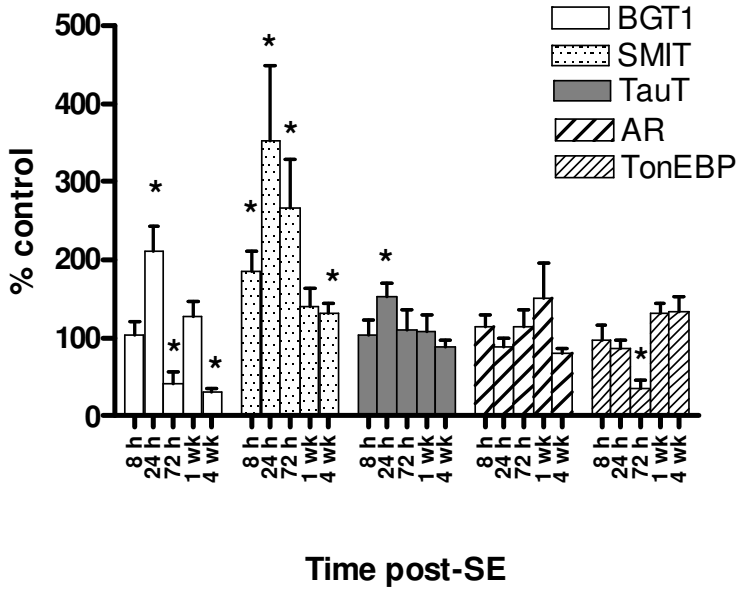
GAT, GABA transporter; SMIT, sodium/myo-inositol cotransporter; TauT, taurine transporter; AR, aldose reductase; TonEBP, tonicity-responsive enhancer binding protein; Psmb6, proteosome subunit beta 6; AT, annealing temperature; PS, expected produce size of the PCR amplicon; bp, base pairs.

Figure 2.1: Alterations in GAT (A) and osmoprotective (B) gene mRNA expression 8 h, 24 h, 72 h, 1 wk, and 4 wks postSE (means ± SEM). Results are expressed relative to control values (normalized to 100%). *= $p<0.05$ vs. control; n = 6-10 per group.

A


BGT1
GAT1
GAT3
% control
250
200
150
100
50
0
8 h
24 h
72 h
1 wk
4 wk
Time post-SE

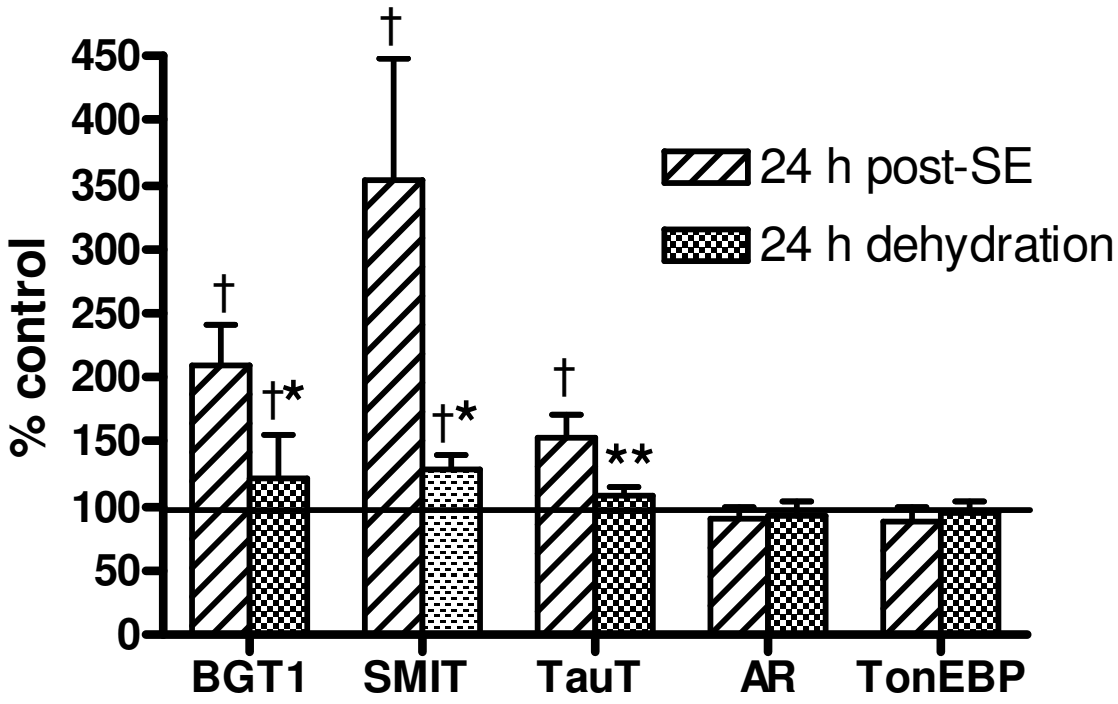
B


BGT1
SMIT
TauT
AR
TonEBP
% control
500
400
300
200
100
0
8 h
24 h
72 h
1 wk
4 wk
Time post-SE

affected by SE at any of the time-points examined. The transcription factor of the osmolyte-related genes, TonEBP, was downregulated 72 h post-SE, and was not altered at other time-points. As such, there is no apparent relationship between the expression of the osmolyte-related genes and TonEBP post-SE. Interestingly, no gene investigated was different from control values one week following SE.

BGT1 mRNA expression appears to be regulated similarly to the osmolyte transporters 24 h post-SE. Since osmolyte transporters may be subject to regulation by the systemic osmotic state, dehydration due to intense motor activity during SE could contribute to the observed upregulation of TauT, SMIT, and BGT1. Animals were significantly dehydrated following both SE and 24 h of water withdrawal compared to controls ($p<0.01$ for both groups; control = 313.3 ± 0.99 mOsm/kg; 24 h post-SE = 326.4 ± 3.1 mOsm/kg; 24 h water withdrawal = 327.7 ± 1.3 mOsm/kg). Plasma osmolality values between experimental groups were strikingly similar. Therefore, the expression profiles of these two groups were compared to determine the impact of dehydration on the expression patterns seen post-SE. As shown in Figure 2.2, SE animals experienced significantly greater alterations in osmoprotective genes than those observed in dehydrated animals. In fact, only SMIT was significantly increased following 24 h of water withdrawal. This is not surprising, because a mild dehydration protocol was chosen for this study to produce similar plasma osmolality values to those seen 24 h post-SE. These results suggest that the alterations in osmolyte

Figure 2.2: Effect of SE (24 h postinsult) and 24 h water withdrawal on osmoprotective gene mRNA expression (means ± SEM). Results are expressed relative to control values (normalized to 100%). BGT1, TauT, and SMIT mRNA are significantly higher post-SE than postdehydration. †= $p<0.05$; ††= $p<0.01$ vs. control; *= $p<0.05$ vs. post-SE; ** = $p<0.01$ vs. post-SE; n = 8-10 per group.


% control
450
400
350
300
250
200
150
100
50
0
†
†*
†
†*
†
**
24 h post-SE
24 h dehydration
BGT1
SMIT
TauT
AR
TonEBP

transporter mRNA expression following SE are not accounted for by dehydration.

BGT1 mRNA expression is downregulated 72 h and 4 weeks post-SE, similarly to the GABA transporters, GAT1 and GAT3. The effect of an inflammatory insult on the downregulation of these genes was investigated. LPS-induced inflammation was confirmed by a significant increase in body temperature ($p<0.01$; control= -0.01 ± 0.07 ℃; LPS= 1.21 ± 0.29 ℃) and weight loss ($p<0.01$; control= -0.1 ± 0.1 g; LPS= -3.3 ± 0.1 g) compared to controls. Plasma osmolality was not different between groups. As shown in Figure 2.3, GAT1 and GAT3 mRNA expression were decreased in response to inflammation, while BGT1 mRNA expression was not altered. Inflammation may therefore contribute to the regulation of GAT1 and GAT3. As seen in Figure 2.4, BGT1, TauT, SMIT, and AR mRNA levels were not altered post-LPS. TonEBP, however, was significantly increased by exposure to LPS. These results illustrate that, despite being a known transcription factor of the investigated osmoprotective genes, TonEBP does not appear to activate these genes in response to the specific inflammatory stimulus utilized in this investigation.

Discussion

Osmoprotective gene expression is altered by tonicity due to promotion by the transcription factor TonEBP. Under osmotic stress, TonEBP expression can increase and alter the expression of BGT1, TauT, SMIT, and AR (Woo et al. 2002). SE results in dehydration, and it is possible that the changes in gene

Figure 2.3: Effect of SE (4 weeks postinsult) and LPS (24 h postinsult) on GAT mRNA expression (means ± SEM). Results are expressed relative to control values (normalized to 100%). GAT1 and GAT3, but not BGT1, are altered postLPS. †= $p<0.05$; ††= $p<0.01$ vs. control; *= $p<0.05$ vs. post-SE; n = 6-10 per group.

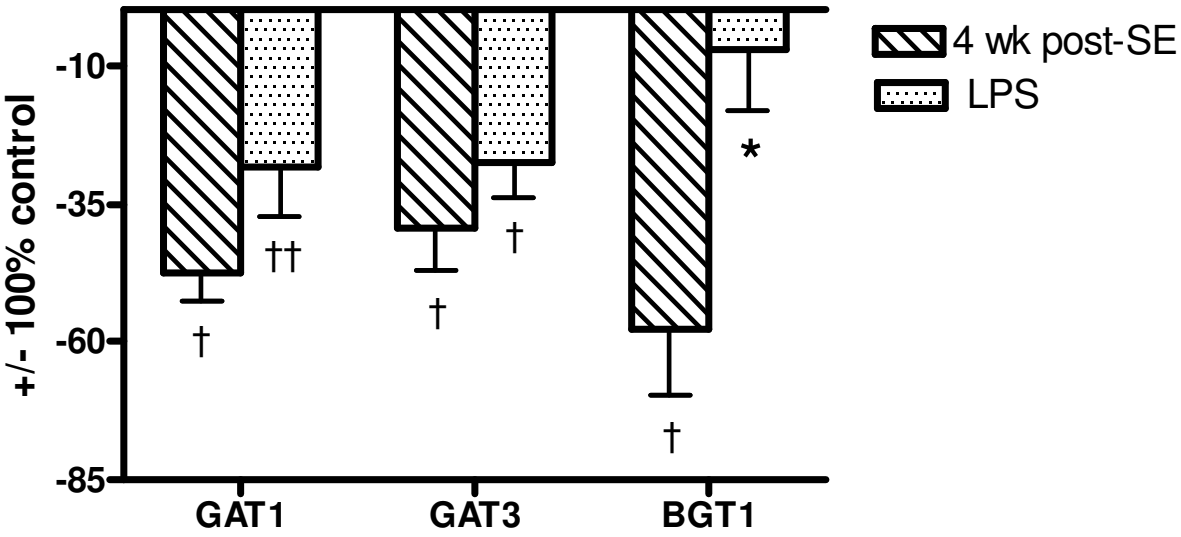
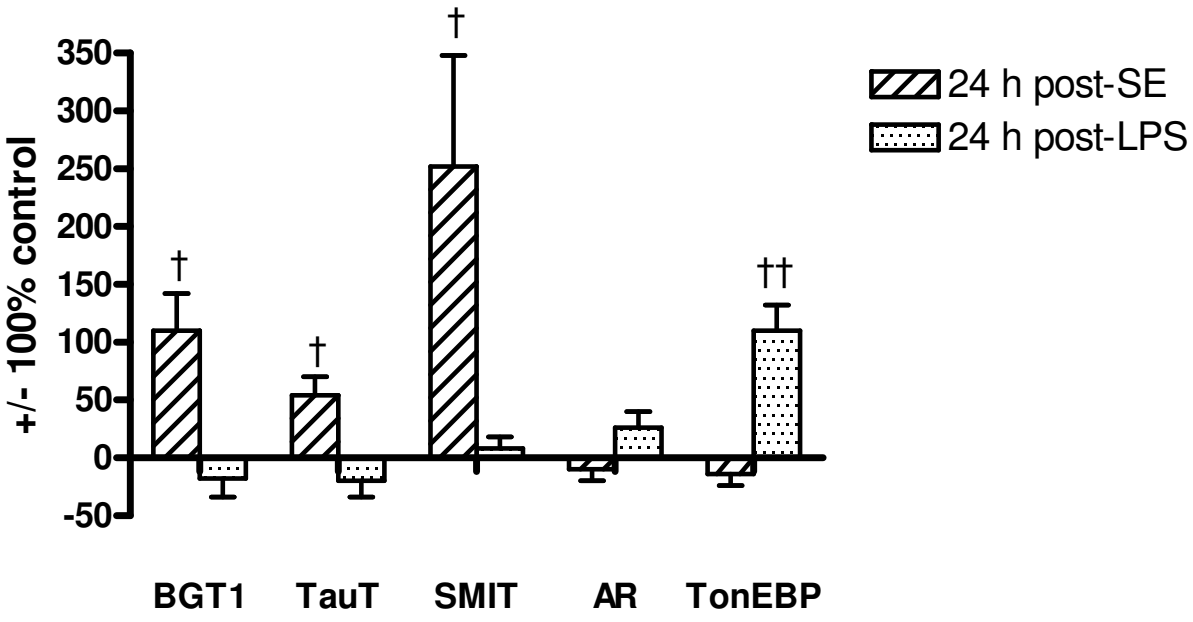

4 wk post-SE
LPS
+/- 100% control
-10
-35
-60
-85
GAT1
GAT3
BGT1
†
††
†
†
*
†

Figure 2.4: Effect of SE (24 h postinsult) and LPS (24 h postinsult) on osmoprotective gene mRNA expression (means ± SEM). Results are expressed relative vehicle controls (normalized to 100%). BGT1, TauT, and SMIT mRNA are altered post-SE while TonEBP is altered post-LPS. †= $p<0.05$; ††=0.01 vs. control; n = 8-10 per group.


+/- 100% control
350
300
250
200
150
100
50
0
-50
†
†
†
††
BGT1
TauT
SMIT
AR
TonEBP
24 h post-SE
24 h post-LPS

expression observed in osmoprotective genes following SE were due to dehydration. Following SE, animals were dehydrated to a similar extent to animals that had water withheld for 24 h as determined by plasma osmolality values. However, SE produced upregulation of BGT1, TauT, and SMIT mRNA, while 24 h water withdrawal caused exclusive upregulation of SMIT mRNA. SMIT, TauT, and BGT1 mRNA values following 24 h water withdrawal were significantly lower than the expression values following SE. This result indicates that dehydration does not account for the upregulation of the osmolyte transporters following SE.

BGT1 expression has been shown to closely mimic TonEBP expression in the kidney, but an apparent disparity exists between their expression patterns in the brain (Zhang et al. 2003; Maallem et al. 2006b). In agreement with these previously reported trends, the current study also found that osmolyte-related genes do not mimic TonEBP expression in the brain following SE, mild dehydration, or inflammation. While SMIT, TauT, and BGT1 mRNA were increased 24 h post-SE, TonEBP mRNA was unaltered at this time-point. TonEBP and BGT1 mRNA were significantly downregulated at 72 h post-SE, while SMIT was upregulated and TauT was not different from control values. At 4 weeks post-SE, SMIT was increased and BGT1 was decreased, while TonEBP and TauT were not different from controls. Thus, it appears that the mRNA expression patterns of the osmoprotective genes are not following those of TonEBP in the brain. Furthermore, they also appear to be regulated differentially from each other. In contrast to TonEBP, AR mRNA was not altered by any

manipulation performed in the current study, i.e., SE, mild dehydration, or inflammation. This is not surprising because of the determined discrepancy between expression of these genes in the brain, and because a mild dehydration protocol was utilized. From the currently available data, it appears that the osmolyte transporters are not exclusively regulated by TonEBP in the brain.

Inflammation was thought to contribute to the alterations in GATs and osmoprotective genes because both GAT1 (Wang et al. 2008) and TonEBP (Trama et al. 2000) have been previously associated with inflammation. Furthermore, seizures induce rapid yet persistent CNS inflammation which can enhance neuronal excitability, reduce cell survival, and increase blood-brain barrier permeability, resulting in a predisposition to seizure activity (see (Vezzani and Granata 2005) and (Choi and Koh 2008) for reviews). Therefore, mRNA values observed post-SE were compared to those obtained from mice that had been subjected to an inflammatory insult to determine whether inflammation could contribute to the downregulation seen post-SE. GAT1 and GAT3 values were decreased post-LPS compared to controls, and were not significantly different from SE animals. This finding supports the hypothesis that inflammation may contribute to GAT downregulation post-SE; however, it appears that GAT downregulation post-SE is more severe than post-LPS, suggesting that other mechanisms contribute to the observed post-SE GAT downregulation. Further studies will be necessary to determine the nature and extent of inflammation post-SE, and to determine the degree to which inflammation may actually contribute to the observed GAT downregulation.

TonEBP (NFAT5) is induced by T-cell activation, but AR is not increased by this receptor-mediated stimulus (Trama et al. 2000). The current study complements these data, and is the first to investigate inflammatory-mediated regulation of TonEBP in the brain, as well as its downstream targets. The results demonstrate that TonEBP is responsive to neuroinflammation but does not regulate osmotic elements under the specific conditions investigated. BGT1, TauT, and SMIT were significantly altered post-SE, but were not changed post-LPS. Hence, inflammation does not contribute to the transcriptional response of BGT1 and the osmolyte transporters under the investigated conditions. Surprisingly, TonEBP was not upregulated following SE, despite the known occurrence of an inflammatory response following seizures. It is possible that TonEBP is regulated by only a subset of inflammatory stimuli, e.g., TonEBP responded to LPS, which signals through Toll-like receptor 4 (Poltorak et al. 1998). The innate and/or adaptive inflammatory mediation following seizures may not activate pathway(s) that activate TonEBP expression, although Toll-like receptor 4 has been implicated in ictogenesis (Maroso et al. 2010).

The observed expression patterns closely mirror the phases of the pilocarpine-induced SE model of temporal lobe epilepsy. This model includes an acute insult that produces seizure activity, followed by a recovery period during which animals have a low probability of seizures (Williams et al. 2007), followed by a chronic period when spontaneous seizure activity occurs (Leite et al. 2002). The phase of low seizure probability is referred to as the latent period, and is the stage in which the cellular and molecular alterations occur that underlie the

eventual development of spontaneous seizures, i.e., epileptogenesis (Loscher 1998; White 2002). Approximately 2 weeks following SE (13.1 ± 2.4 d in C57/B6 mice, (Shibley and Smith 2002)), animals begin to display recurrent spontaneous seizure activity, although monitoring was not performed in the current study. The observed genes were altered in the acute and long-term phases of the model, but returned to normal during the latent period. The functional significance of the current findings remains to be determined. Since GATs are able to reverse and release GABA into the extracellular environment (Attwell et al. 1993; Levi and Raiteri 1993), the net effect of their upregulation is unclear.

The results obtained in this investigation are similar to previous studies. SMIT was increased following kainic acid-induced SE, and peaked in CA1 of the hippocampus at 24 h post-SE before returning to normal (Nonaka et al. 1999). The study was only carried out for 1 week, so a long-term comparison cannot be made with this study. Kainic acid-induced SE also caused a downregulation of GAT1 and GAT3 mRNA. GAT1 was significantly downregulated at 24 h and 30 d post-SE, but returned to normal at 1-week post-SE. These results are in close agreement with those found in the current study. GAT3, however, remained consistently downregulated following kainic acid-induced SE, in contrast to the results obtained with pilocarpine-induced SE in this study (Sperk et al. 2003). As such, it is important to keep in mind that different results may be observed in other epilepsy models compared to those currently observed in the pilocarpine model.

In summary, BGT1 mRNA expression is upregulated early post-SE along with other osmolyte transporters; at later time-points, BGT1 is downregulated along with the GABA transporters. TonEBP does not appear to exert full control over the expression of BGT1 and the other osmoprotective genes in the brain, nor does it induce their expression in response to acute LPS-induced inflammation. Neither plasma osmolality nor inflammation appear to account for the changes observed in BGT1/osmolyte transporter mRNA expression post-SE. In contrast, inflammation may contribute to the downregulation of GAT1 and GAT3. The mediator(s) of BGT1/osmoprotective mRNA regulation in the brain post-SE therefore remain(s) unknown. However, it is evident that these genes are altered by seizure activity. Since osmotic balance plays a role in controlling neuronal excitability, further investigation into the regulation of, and the role played by these genes in epilepsy is warranted. BGT1 is of particular interest due to its ability to transport both GABA and the organic osmolyte betaine.

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CHAPTER 3

COMPARATIVE ANTICONVULSANT EFFICACY IN THE CORNEAL KINDLED MOUSE MODEL OF PARTIAL EPILEPSY: CORRELATION WITH OTHER SEIZURE AND EPILEPSY MODELS

Nicole M. Rowley and H. Steve White (2010) Comparative anticonvulsant efficacy in the corneal kindled mouse model of partial epilepsy: Correlation with other seizure and epilepsy models.

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Comparative anticonvulsant efficacy in the corneal kindled mouse model of partial epilepsy: Correlation with other seizure and epilepsy models

Nicole M. Rowley, H. Steve White *

Anticonvulsant Drug Development Program, Department of Pharmacology & Toxicology, University of Utah, 417 Wakara Way, Suite 3211, Salt Lake City, UT 84108, United States

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Summary Chronic electrical stimulation via corneal electrodes can rapidly yield large numbers of kindled mice with a seizure phenotype reflective of secondarily generalized partial seizures. The corneal kindled mouse model has been found to be a highly sensitive and efficient screening model for antiepileptic drug (AED) discovery. The present study further evaluates the utility of the corneal kindled mouse model as a tool for rapid screening of investigational AEDs. Results obtained with nine AEDs (valproic acid, lamotrigine, phenytoin, carbamazepine, levetiracetam, vigabatrin, topiramate, tiagabine, and ezogabine) with varying mechanisms of action and clinical spectrums, as well as six investigational compounds were evaluated in the corneal kindled mouse. ED_{50} values are compared to those obtained in the hippocampal kindled rat, the mouse maximal electroshock (MES) model, the 6 Hz partial psychomotor seizure model, and the subcutaneous pentylenetetrazol (scPTZ) test. The results obtained in the corneal kindled mouse demonstrate a positive correlation with those attained employing established preclinical models: MES ($r^2 = 0.9511$), scPTZ ($r^2 = 0.9697$), 6 Hz ($r^2 = 0.9519$), and hippocampal kindling ($r^2 = 0.9037$). The demonstrated predictive ability of the corneal kindled mouse model supports its use in the early evaluation of investigational AEDs.

Introduction

There are a number of animal models of evoked seizures and epilepsy currently employed in the search for new antiseizure drugs; however, no one model can successfully predict efficacy for every new AED. For this reason, a diverse set of preclinical models is incorporated in the initial screening of candidate compounds (White et al., 2002). The maximal electroshock (MES) seizure model, first utilized in 1937 (Putnam and Merritt, 1937), led to the discovery of the anticonvulsant properties of phenytoin (PHT). Following this discovery, the MES test became established as one of the primary preclinical screening tools utilized in the search for new AEDs. Other acute screening tools commonly used

* Corresponding author. Fax: +1 801 581 4049.
E-mail address: swhite@hsc.utah.edu (H.S. White).

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include the subcutaneous pentylenetetrazol, also known as metrazol, test (scPTZ or scMet) and the 6 Hz partial 'psychomotor' seizure test (White et al., 2002). Unfortunately, clinical results have challenged the predictive nature of both the MES and the scPTZ acute seizure models. For example, levetiracetam (LEV) was found to be inactive in the MES and scPTZ screens. In contrast, LEV was active in the rat amygdala kindling seizure model (Löscher et al., 1998), a chronic model of epilepsy. LEV is currently a clinically utilized AED with a novel mechanism of action; i.e., it binds with high affinity to a synaptic vesicle protein (SV2A) (Lynch et al., 2004). The inability of the primary screens to identify the anticonvulsant efficacy of LEV raised significant concerns regarding their capacity to identify drugs with novel mechanisms of action. The MES screen also failed to detect the efficacy of tiagabine (TGB) and vigabatrin (VGB), two clinically efficacious AEDs that work to increase synaptic GABA levels, albeit via different mechanisms. Furthermore, the MES model may determine efficacy that is not reproducible in humans. For example, NMDA antagonists are very effective in the MES screen, but their efficacy in human patients with epilepsy could not be demonstrated at doses that were devoid of psychiatric complications (Löscher and Hönack, 1991). The 6 Hz seizure test is non-discriminating at low stimulus currents and can be used as a screen to identify potential AEDs. As the stimulus intensity is increased, it becomes highly discriminating and can be used to differentiate an investigational drug from those currently available for the treatment of partial epilepsy. For example, at high stimulus intensities, PHT, carbamazepine (CBZ), lamotrigine (LTG), and topiramate (TPM) are all inactive at non-toxic doses. In contrast, with the exception of TPM, all of these compounds are active at lower stimulus intensities (Barton et al., 2001). Also, LEV is active in the 6 Hz test, while it is inactive in the other primary screens; e.g., the MES and scPTZ tests. Thus, the 6 Hz model is useful for differentiating the pharmacological profile of investigational compounds. The ideal preclinical model, as described by the National Institutes of Health (NIH)/NINDS/American Epilepsy Society (AES) Models II Workshop (2002), (1) would display spontaneous seizures following an appropriate post-insult latent period, (2) would be resistant to at least two existing AEDs, (3) would be amenable to high throughput screening, and (4) would reflect a pathophysiology and phenomology similar to human epilepsy (Stables et al., 2003). While the MES and scPTZ screens are amenable to high throughput screening, they do not meet any of the remaining criteria. The 6 Hz model possesses a differential pharmacological profile compared to MES and scPTZ tests. In this respect it can serve as a potential screen for pharmacoresistant epilepsy. However, the seizures are still acutely evoked and involve a normal animal, not an epileptic animal. The kindled rat model of partial epilepsy is a model of chronic hyperexcitability; however, it is not particularly amenable to high throughput screening and animals do not routinely display spontaneous seizures. Nonetheless, electrical kindling (focal seizures) in rats is a commonly used chronic model for temporal lobe epilepsy and is highly predictive of AEDs with clinical efficacy for partial seizures (Smith et al., 2007). In addition, the kindled rat offers a more informative look into the epileptic condition than do the naïve, pathologically normal animals utilized in acute primary screens. The advantage of using ''seizure experienced'' animals is that the pharmacology of AEDs can be altered by the epileptic pathology (Honack and Loscher, 1995). A good example of this is the lack of observed efficacy of LEV when evaluated in models of acute evoked seizures, such as the scPTZ and MES models, vs. the marked efficacy observed in the kindled rat model.

The corneal kindled mouse model possesses certain advantages as a primary screening model compared to the hippocampal kindled rat. While possessing increased clinical predictability, most electrical kindling models are limited by many factors, which render them impractical as primary screens. First, a significant amount of time is required to prepare and care for implanted rats. Second, a great deal of skilled labor is invested in stereotaxic surgeries, post-operative care, and kindling. Third, the means necessary to financially support the required resources and staff are limiting. Finally, because of the age and body mass of the rats utilized, a large amount of compound is required for dosing. The model therefore possesses both time- and labor-intensive preparation requirements (electrode implantation surgery and a recovery period in addition to the time required to conduct a study in a chronic model), and the cost of the model is high not only due to time and labor constraints but also to the chronic housing and feeding of multiple adult rats. Corneal kindling in mice has been proposed as a cost-effective alternative model for screening compounds for the treatment of partial epilepsy (Sangdee et al., 1982; Matagne and Klitgaard, 1998). The optic nerve is positioned such that the brain may be transcorneally stimulated, creating a non-invasive route for rapid kindling. In addition, the smaller animal size minimizes the feeding, housing, and dosage requirements that limit the usefulness of the electrically kindled rat model.

Methods

Animals

Adult male CF1 mice weighing a minimum of 18 g (Charles River, Kingston, WA) or male Sprague—Dawley rats weighing approximately 250 g at the onset of kindling (Charles River, Raleigh, NC) were used to determine ED_{50} values in each of the paradigms described. Animals were housed in a temperature-, humidity-, and light-controlled (12 h light:dark cycle) facility. Mice were group housed and rats housed individually and permitted free access to food and water. All experimental procedures were performed in accordance with the guidelines established by the National Institutes of Health (NIH) and received approval from the University of Utah's Animal Care and Use Committee (IACUC).

Corneal kindling (mice)

Mice were kindled according to the optimized protocol defined by Matagne and Klitgaard (1998). Briefly, mice were stimulated twice daily with a corneal stimulation of 3 mA (60 Hz) for 3 s for an average of 12 days. Prior to each stimulation, a drop of 0.9% saline containing 0.5% tetracaine hydrochloride (Sigma—Aldrich, St. Louis, MO) was applied to the cornea to ensure local anesthesia and good electrical conductivity. Stimulations were delivered 4 h apart. Animals were considered kindled when they displayed five consecutive stage five seizures according to the Racine scale (Racine, 1972). At the completion of the kindling acquisition, mice

were permitted at least a 10-day stimulation-free period prior to any drug testing. Mice were stimulated once the day before drug testing to ensure they had achieved and maintained a kindled state.

Compound screening (corneal kindled mice)

On the day of the drug study, groups of mice ($n = 8$) received an intraperitoneal (i.p.) injection of the test AED. At the previously determined time to peak effect (TPE) for each AED, motor function was evaluated using the rotarod test (Dunham and Miya, 1957), after which mice were immediately challenged with the corneal kindling stimulus of 3 mA for 3 s. Mice were scored as protected (seizure score of ≤ 3) or not protected (seizure score ≥ 4), based on the Racine scoring system (Racine, 1972). The dose was increased or decreased between the limits of 0 and 100% protection until sufficient data had been obtained to calculate the median effective dose (ED_{50}). A 3-day washout period was permitted between each challenge drug dose. Results were compared to those obtained in established preclinical screening models (MES, scPTZ, 32 mA 6 Hz, and hippocampal kindling models).

Electrode implantation (rats)

Sprague—Dawley rats were anesthetized with a single cocktail containing 120 mg/kg ketamine hydrochloride (100 mg/ml) and 12 mg/kg xylazine (100 mg/10 ml), administered i.p. A maintenance dose (60 mg/kg, i.p.) was given as needed. Animals were placed in a stereotaxic device and electrodes implanted at AP − 3.6, ML 4.9, DV − 5.0 from dura, and incisor bar +5 (Lothman et al., 1988). Dental resin was applied, followed by Triple Antibiotic Ointment, buprenorphine (0.02 mg/kg, s.c.) and Bicillin (60,000 units, s.c.) before animals were returned to their cages for a 1-week post-surgical recovery period.

Hippocampal kindling (rats)

Rats were kindled according to the protocol established by Lothman et al. (1988). Briefly, rats were stimulated with suprathreshold trains of 200 μA for 10 s at 50 Hz every 30 min for 6 h (12 stimulations per day) on alternate days (4—5 stimulus days) until fully kindled.

Drug screening (rats)

One week after reaching a fully kindled state of four or more stage 4/5 seizures, the effect of a single high dose of the test substance administered i.p. was assessed by determining its effect on the after discharge duration and behavioral seizure score (BSS) using the Racine scoring system (Racine, 1972). A single group of kindled rats ($n = 6$—8) was tested at 15, 45, 75, 105, 135, 165, and 195 min after drug administration. Results obtained at the various time points were compared with the last control stimulus, delivered 15 min prior to drug administration, so each animal served as its own control. Previous investigations have found that repeated hippocampal stimulation of animals does not negatively alter seizure threshold in the hippocampal kindled rat model. If protection was observed (stage 3 seizure or less) a dose—response study was initiated. The dose was varied between 0 and 100% protection and an ED_{50} was calculated. Animals were used serially, and allowed a 4—5-day washout period between experiments.

Maximal electroshock test (mice)

A drop of 0.5% tetracaine hydrochloride was placed on the eyes when the mouse received the test compound. At the previously determined TPE, a drop of electrolyte solution (saline) and the electrodes were placed on the eyes. Fifty mA (60 Hz) of alternating current was delivered via corneal electrodes for 0.2 s using an apparatus originally designed by Woodbury and Davenport (1952). This supramaximal current is sufficient to evoke a maximal tonic extension seizure in mice. Animals not displaying a full hindlimb tonic extension were considered protected.

Subcutaneous metrazol seizure threshold test (mice)

At the previously determined TPE for the test compound, PTZ was injected at the CD_{97} (85 mg/kg, s.c.) into a loose fold of skin in the midline of the neck. Mice were placed in Plexiglas isolation chambers ($3'' \times 3'' \times 4''$) and observed for 30 min for the presence or absence of a clonic seizure (approximately 3—5 s episode of clonic spasms of the fore and/or hindlimbs, jaws, or vibrissae). Animals not displaying this phenotypic behavior were considered protected.

6 Hz ''psychomotor'' seizure test (mice)

At the previously determined TPE, mice were challenged with a corneal stimulation of 32 mA for 3 s (6 Hz). Mice not displaying a seizure characterized by a minimal clonic phase followed by stereotyped, automatistic behaviors such as jaw chomping or vibrissae twitching were considered protected.

Anticonvulsant drug preparation

All AEDs were suspended in 0.5% methyl cellulose (Sigma—Aldrich, St. Louis, MO) and sonicated for 15 min prior to administration to ensure complete dissolution or a micronized suspension. Prototype AEDs including CBZ, LTG, PHT, and valproic acid (VPA) were purchased from Sigma—Aldrich, St. Louis, MO, while LEV and VGB were kindly provided by UCB Pharma (Braine, Belgium) and the National Institute of Neurological Disorders and Stroke, respectively. Ezogabine (EZG) was kindly provided by Valeant Pharmaceuticals, Costa Mesa, CA. TPM was kindly provided by Johnson and Johnson Pharmaceutical Research and Development (Springhouse, PA), and TGB was provided by Cephalon, Inc. (Frazer, PA). In addition, results obtained with six investigational compounds designated A—F (NIH/NINDS Anticonvulsant Screening Project) were included in the correlation analyses.

ED_{50} determination and statistical analysis

The proportion of responders was calculated as a percent of total animals. Seizure severity was expressed as mean ± S.E.M. for each group. The ED_{50} and 95% confidence interval (CI) for each compound was calculated using a log-probit analysis (Finney, 1971). Additional doses of each AED were administered until the 95% CI was inside the dose range evaluated. Correlations were determined utilizing linear regression (Prism Software, Version 4.02, Graphpad, La Jolla, CA). It should be noted that ADD compound F was excluded from the correlation analyses shown in Fig. 2A and D. Compound F was tested as an outlier by submitting the ratio of the ED_{50} in the corneal kindled mouse model to the ED_{50} in the other tests to the Grubbs extreme studentized deviate (ESD) test for outliers (QuickCalcs Software, Graphpad, La Jolla, CA). Compound F was determined to be an outlier only in the correlation between the corneal kindled mouse model and the hippocampal kindled rat model. The point corresponding to compound F is shown in all panels of Fig. 2, and is labeled in Fig. 2A and D where it was excluded from the calculation of the displayed r^2 value. r^2 values for the hippocampal kindling and MES correlations with and without compound F included are provided in the results section.

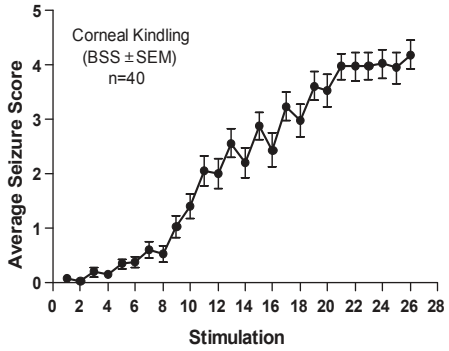

Figure 1 Kindling curve. Male CF1 mice weighing at least 18 g were corneal kindled. Kindling took on average 12 days with twice daily stimulations of 3 mA for 3 s, delivered at least 4 h apart. Seizures were scored according to the Racine scale. Data points are average seizure score ± S.E.M. ($n = 40$).

Results

In the present study, 89.5% (34/38) of mice acquired a fully kindled state; i.e., 5 consecutive stage 5 seizures. As shown in Fig. 1, the kindling curve plateaus at an average seizure score of 4 after 26 stimulations due to the fact that the latency for some mice to reach a fully kindled state was prolonged an additional 12 days. Furthermore, at this point many animals were fully kindled and hence not receiving stimulations, thereby lowering the average seizure score. Of the 38 mice that entered the study, 10.5% (4/38) never achieved a stage 5 seizure. These animals were not utilized for compound screening. No mortality was observed during the kindling process, and the mortality rate throughout the course of compound screening was 15.8% (6/38), with all deaths unobserved during the night.

With the exception of TPM, all of the AEDs tested displayed a dose-dependent protection against secondarily generalized seizures in the corneal kindled mouse model. Overall, the rank order potency obtained with prototype AEDs was TGB > EZG > LTG > LEV > PHT > CBZ > VGB > VPA. An ED_{50} could not be calculated for TPM; a maximum of 50% protection was achievable at 58.9 μmol/kg. The rank order of potency in the corneal kindled mouse matched that reported for the Frings model of reflex epilepsy, a high-throughput screening tool (TGB > LTG ≥ PHT ≥ TPM > CBZ > VPA) (White et al., 2002). Importantly, the corneal kindled mouse model was the only model to demonstrate efficacy of all tested prototype AEDs at non-toxic doses. As shown in Table 1, PHT was active at non-toxic doses in the corneal kindled mouse and the MES test. ED_{50}s for LTG, PHT, TPM, and CBZ could not be determined in the scPTZ test due to toxicity at the highest doses evaluated. VGB and TGB were not active in the MES model. LEV was ineffective in both the MES and scPTZ tests. Furthermore, LEV was not able to provide full protection in the electrical kindled rat. The corneal kindling model identified VGB, TGB, and LEV as active compounds. TPM was inactive in the electrical kindled rat, 6 Hz, and scPTZ seizure models.

The rank order potency of AEDs in the hippocampal kindled rat was TGB > LTG > CBZ > VPA. In the 32 mA 6 Hz seizure

Table 1 ED_{50} values (μmol/kg, i.p.) and (95% CI) are shown for all epilepsy screens performed: corneal kindled mouse, hippocampal kindled rat, 6 Hz, scPTZ, and MES. Compounds A–F are investigational compounds. EZG, ezogabine; LTG, lamotrigine; CBZ, carbamazepine; VPA, valproic acid; LEV, levetiracetam; VGB, vigabatrin; TPM, topiramate; TGB, tiagabine; and PHT, phenytoin. (–): test has not been performed; (>x): toxicity observed at doses that were not fully efficacious and thus precluding the calculation of an ED_{50}. (<x): lower doses were not tested.

Drug	Corneal kindled mouse	Hippocampal kindled rat	6 Hz (32 mA, mice)	scPTZ (mice)	MES (mice)
EZG	24.1 (16.2–34.0)	–	140.1 (105.8–176.7)	44.5 (30.3–59.3)	30.7 (20.8–43.2)
LEV	57.0 (21.2–114.0)	Max protection 50% at 352.5	194.5 (58.2–477.1)	No activity	No activity
LTG	37.1 (22.6–54.7)	61.3 (33.6–110.1)	100.4 (79.7–121.4)	>164.0	33.2 (26.6–44.5)
PHT	66.9 (45.8–97.1)	>119.4	>238.8	>199.0	26.7 (21.5–33.4)
TPM	Max protection 50% at 58.9	No activity	No activity	No activity	97.2 (48.0–171.2)
TGB	0.73 (0.46–1.04)	17.2 (11.2–26.5)	1.6 (0.41–3.0)	0.63 (0.34–1.09)	No activity
CBZ	143.5 (109.6–173.5)	119.4 (69.4–195.5)	109.2 (69.8–157.0)	>190.5	73.2 (57.6–87.2)
VGB	308.1 (122.3–738.6)	–	–	–	No activity
VPA	728.0 (509.6–1016.8)	1287.5 (882.0–1660.6)	840.5 (555.9–1188.9)	1323.6 (1064.3–1610.0)	1582.3 (1425.3–1697.9)
A	0.15 (0.03–0.25)	0.5 (0.25–0.75)	<0.08	0.05 (0.04–0.06)	>45.1
B	4.8 (3.7–6.3)	12.6 (9.3–15.2)	17.8 (15.6–21.5)	31.2 (22.6–51.6)	24.8 (21.9–27.4)
C	13.6 (11.6–17.6)	5.0 (3.0–8.3)	18.3 (12.9–21.9)	–	–
D	282.6 (158.8–599.7)	143.7 (100.4–209.1)	No activity	430.4 (376.1–482.4)	670.4 (598.6–737.6)
E	246.5 (155.0–338.4)	212.0 (166.8–275.0)	–	341.4 (277.0–395.5)	255.8 (238.1–281.9)
F	513.1 (316.5–730.4)	94.8 (52.8–152.6)	–	685.2 (341.0–1305.7)	173.3 (127.5–232.4)

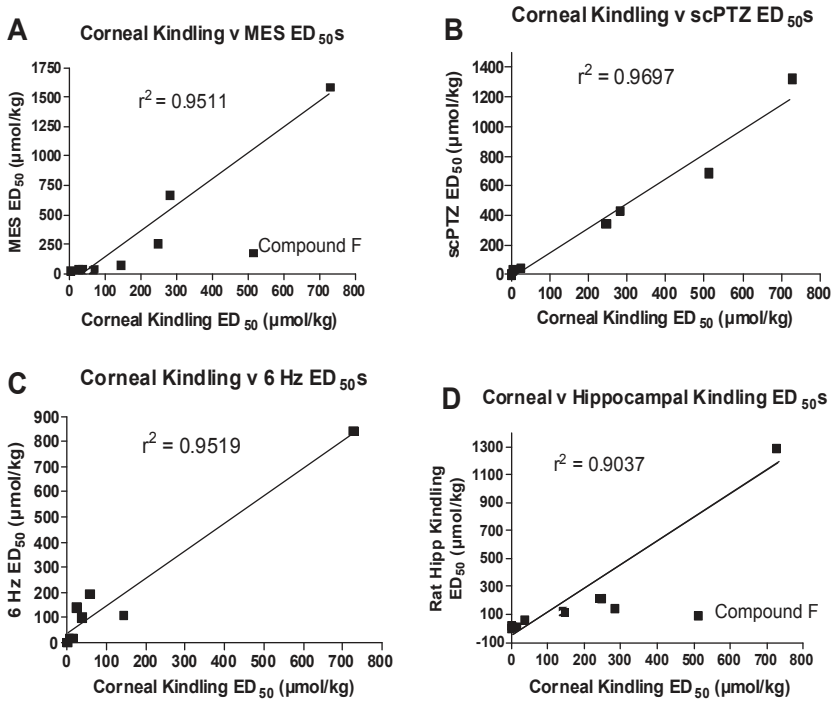

Figure 2 Paradigm correlations. Corneal kindled mouse ED_{50} (μmol/kg, i.p.) results were correlated with ED_{50} (μmol/kg) results from the MES (A), scPTZ (B), 6 Hz (C), and hippocampal kindled rat (D) screens. Each data point represents the ED_{50} of a drug in the corneal kindled mouse model vs. its ED_{50} in the other seizure models. Compound F was excluded from the correlation analyses (r^2 values) shown for the MES and rat hippocampal kindling correlations. See section ED_{50} determination and statistical analysis for details.

test it was TGB > LTG ≥ CBZ > EZG > LEV > VPA, in the scPTZ test it was TGB > EZG > VPA, and in the MES test it was PHT ≥ EZG ≥ LTG > CBZ > TPM > VPA. The ED_{50} values obtained in the various models for all compounds tested are summarized in Table 1.

A strong positive correlation between the effective doses in the corneal kindled mouse model and the hippocampal kindled rat, MES, 6 Hz, and scPTZ screens was demonstrated. The r^2 values for these correlations were 0.9037, 0.9511, 0.9519, and 0.9697, respectively. For the hippocampal kindling and MES test results, the value of ADD compound F was excluded from the correlation coefficient shown in Fig. 2, but is plotted on the correlation graph. Had this value been included in the correlation calculation, the r^2 value would have been 0.6546 for the hippocampal kindling model and 0.6888 for the MES model. Since VPA has a much larger value than the other compounds, it may appear that the positive correlations depend heavily upon the inclusion of VPA. However, in the absence of VPA, a positive correlation remains between the corneal kindled mouse model and the other model systems. Without VPA, the r^2 values for the correlations are 0.7404, 0.995, and 0.8733 for the MES, scPTZ, and hippocampal kindled rat models, respectively. With the removal of only VPA, the 6 Hz correlation drops to 0.2515 due to a now poorly fitting value of CBZ. Following the removal of VPA and CBZ, the r^2 value for the 6 Hz correlation is 0.8442.

Discussion

The corneal kindled mouse model quickly and easily yields a population of chronically kindled animals available for serial use in the initial screening of AEDs. The use of this model is preferred as an initial screen to the electrical kindled rat model in that it conserves compound, time, and labor, thereby reducing costs. It also requires less technical skill since no surgical expertise is required. The corneal kindled mouse model is advantageous over the other primary screens, including the 6 Hz test, the MES test, and the scPTZ test, because it displays a pharmacological profile consistent with human partial epilepsy. Moreover, it effectively identified the anticonvulsant potential of compounds that bind to SV2A, such as levetiracetam, brivaracetam and seleteracetam (Rogawski, 2006). The scPTZ and MES tests did not detect the anticonvulsant potential of LEV, while the corneal kindled mouse and 6 Hz models did. PHT was not fully active in the 6 Hz and scPTZ models and VGB and TGB were not active in the MES test. TPM was not active in the hippocampal kindled rat, 6 Hz, or scPTZ seizure models. The corneal kindled mouse model, however, was able to successfully identify the anticonvulsant activity of PHT, VGB, TGB, and TPM. The results obtained in this study confirm and emphasize the suggestion that reliance on acute, evoked seizures for early preclinical testing is inadequate for fully determining the anticonvulsant potential of novel compounds.

The results obtained in the corneal kindled mouse model correlated well with those obtained in the hippocampal kindled rat. Furthermore, the corneal kindled mouse was able to detect efficacy for all clinically active compounds tested. However, the results obtained in this investigation are not intended to suggest that it would fully replace the electrical kindled rat model. While mice can be utilized for long periods of time if they are routinely stimulated, they have been reported to lose their kindled state after a 4-week stimulation-free period. Furthermore, the altered sensitivity to adverse effects also disappears after a 27-day stimulation-free period in the corneal kindled mouse (Potschka and Löscher, 1999). These results were not observed in the current study because animals did not receive long stimulation-free periods. The reported loss of kindling and adverse effect profile demonstrate that brain alterations are not permanent in the corneal model, while they appear to be in the electrical kindled rat. The efficacy of kindling can be manipulated by stimulus intensity, duration, and the interval between stimuli. These parameters were previously determined and optimized (Sangdee et al., 1982; Matagne and Klitgaard, 1998). Therefore, altering the kindling parameters does not easily ameliorate the aforementioned minor drawbacks of the model. Various mouse strains respond differently to kindling (kindling rate, mortality rate, etc., unpublished observations). In the future, an optimal strain may be determined for the use of this model. However, for the current study it was desirable to utilize the same mouse strain that is utilized for the other primary screens in order to avoid the confounding factor of strain differences.

One distinct disadvantage of the corneal kindled mouse model is that, unlike the electrical kindled rat, there is no electroencephalograph (EEG) available since electrodes are not implanted. As such, the effect of an investigational drug on focal seizure activity can only be inferred from behavioral seizure data. In addition, the ideal epilepsy model would generate spontaneous behavioral seizures; while kindled animals can develop spontaneous seizures after long periods of time in an ''over-kindled'' state (Brandt et al., 2004), this is not a practical utilization of this particular model for compound screening. Another possible disadvantage of the corneal kindled mouse model is that a high mortality rate during kindling (22.5% in males) has been reported (Potschka and Löscher, 1999). However, in the current study there was no mortality observed during the kindling process. In the limited scope of this study, no compound was found to be inactive in the corneal kindled mouse, although TPM lacked 100% protection. This fact may be viewed in a positive light, in that it confirms the broad spectrum of utility for the corneal kindled model. However, there is a great need for a deeper understanding of pharmacoresistant epilepsy, and, as such, an appropriate model system must be developed. For the corneal kindled mouse to be considered a model of pharmacoresistant seizure activity, it would have to be resistant to at least two existing AEDs. Further investigation is necessary to determine if the corneal kindled mouse has the potential to be a model of pharmacoresistant epilepsy.

In summary, the corneal kindled mouse model requires an initial investment in time to kindle a population of mice for pharmacological testing; however, it provides a ''seizure experienced'' circuit for drug testing and in this regard more closely reflects that seen in patients with epilepsy. The corneal kindled mouse model demonstrated strong positive correlations with several diverse screening models, chronic and acute, and electrical and chemical. Beyond its predictive capacity, the corneal kindled mouse model conforms to certain criteria for the ideal preclinical model described previously: it is amenable to high throughput screening following the initial labor of the kindling process, and also reflects characteristics of human partial epilepsy. The findings of this study suggest that the corneal kindled mouse model of partial and secondarily generalized epilepsy is a sensitive and valid screening model. The early demonstration of efficacy of an investigational AED in the corneal kindled mouse could provide sufficient proof-of-concept in a chronic seizure model to support more extensive studies in the labor-intensive electrical kindled rat.

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CHAPTER 4

PHARMACOLOGICAL PROFILE AND BGT1-SPECIFICITY OF HIT 8

Introduction

GABA is the main inhibitory neurotransmitter in the mammalian central nervous system; it is released from the presynaptic terminal and exerts its effects *via* ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors (Watanabe et al. 2002). GABAergic signaling is terminated by rapid uptake into the surrounding cells by GABA transporters (GATs). The GATs are members of the family of Na^+ and Cl^--dependent transporters (*SLC6* gene family), and include SLC6a1, SLC6a12, SLC6a13, and SLC6a11, termed GAT1-4 (mice) or GAT1, BGT1, and GAT2 and 3 (rat and human) (Schousboe and Kanner 2002). The rat/human nomenclature is suggested by the HUGO Gene Nomenclature Committee and will be used hereafter.

GABA is involved in the modulation of neuronal excitability to manage seizures, pain (Jasmin et al. 2004), and depression (Krystal et al. 2002; Kalueff and Nutt 2007). A specific GAT1 inhibitor, tiagabine (TGB), is Federal Drug Administration (FDA)-approved for the adjunctive treatment of partial epilepsy (Suzdak and Jansen 1995). Although not FDA-approved, TGB has been found useful for various types of pain (Novak et al. 2001; Solaro and Tanganelli 2004;

Kast 2005; Todorov et al. 2005; Landmark 2007), and the negative affect associated with clinical depression (Kalueff and Nutt 2007). Efficacy of TGB in these diverse disorders highlights the potential broad clinical utility of GAT inhibition in treating a variety of neurological disorders. Unfortunately, the use of TGB is limited by its inability to produce seizure freedom, its ability to actually induce seizures in some patient populations, and various side effects (Balslev et al. 2000; Genton et al. 2001). GAT1 is the predominant GAT in the mammalian brain (Jensen et al., 2003; Chen et al., 2004; Conti et al., 2004) and the utility of GAT1 inhibition as a therapeutic target has been thoroughly investigated due to the availability of potent and highly selective GAT1 inhibitors. Unfortunately, less is known about the therapeutic potential of inhibitors that target the other three GAT subtypes due to their lower expression levels and the lack of selective pharmacological inhibitors. Recently, EF1502, a compound that displays equal potency at GAT1 and BGT1, demonstrated that BGT1 inhibition may represent a novel anticonvulsant strategy. EF1502 was shown to be synergistic with TGB in the Frings mouse model of reflex epilepsy, the ivPTZ seizure threshold test, and in the corneal kindled CF1 mouse (White et al. 2005; Madsen et al. 2009). Furthermore, EF1502 was able to reduce spontaneous bursting in an *in vitro* model of epilepsy while TGB did not, suggesting that the BGT1 inhibitory action of EF1502 was partially responsible for this effect (Smith et al. 2008).

GAT inhibitors have historically been developed based on the chemical structure of GABA or other known GAT substrates such as β-alanine and 2,4-diaminobutyric acid. Both TGB and EF1502 were designed with GABA analogs

as scaffolds (nipecotic acid and exo-THPO, respectively (Krogsgaard-Larsen and Johnston 1975; Clausen et al. 2005, 2006)). Most synthesized GAT-targeting compounds have been found to be GAT1 selective, and attempts at synthesizing non-GAT1 inhibitors have largely led to compounds that inhibit all three of the other GATs (Thomsen et al. 1997; Beuming et al. 2006; Clausen et al. 2006). One potential explanation for the lack of selectivity is that the substrate-binding site is relatively well-conserved among the GATs (Beuming et al., 2006). Therefore, utilizing GAT substrates as scaffolds for inhibitor development may predispose to the identification of nonspecific inhibitors. To avoid this, the current study utilized Hit 8 (molecular weight =363.28), a BGT1 inhibitor that was identified in a diverse compound library screen. It is important to note that Hit 8 is not a GAT substrate or GABA analog. The reported GABA IC_{50} value for Hit 8 was 39 µM at mouse BGT1 and 20 µM at human BGT1. In cells transfected with mouse GATs, Hit 8 displayed only minor inhibitory activity at GAT2 and GAT3 (>200 µM) and was inactive at GAT1. In cells transfected with human GATs, Hit 8 displayed minor inhibition of GAT2 (>100 µM) and no activity at GAT3 or GAT1. Hit 8 was concluded to be an allosteric, non-competitive inhibitor that is selective for BGT1 compared to the other GABA transporters (unpublished observations of Bolette Christiansen et al, manuscript in preparation). This library screening approach, as opposed to rational structural design, may aid in the identification of novel structures that are chemically unrelated to GABA and other GAT substrates and hence may display better GAT selectivity than attained utilizing structural design.

The current study was performed to determine if Hit 8 exerts an effect on several GABA-related behaviors including seizure susceptibility, pain, and depression. Furthermore, the BGT1 specificity of Hit 8 was further characterized in order to determine if BGT1 inhibition is likely to be functionally important in the mechanism of action of Hit 8.

Methods

Animal care: Male 129SV, C57/B6, or CF1 mice weighing 18-25 grams were used for this study (129 SV and C57/B6 mice from The Jackson Laboratory, Bar Harbor, ME; CF1 mice from Charles River, Kingston, WA). Male and female Frings mice weighing 18-35 grams obtained from an in-house colony at the University of Utah were also used. Mice were maintained in an AAALAC-approved light, temperature, and humidity-controlled facility with free access to food and water. All procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and under approval from the University of Utah's Institutional Animal Care and Use Committee (IACUC).

In vivo evaluation: Hit 8 was tested for efficacy in several seizure models and the forced swim test, a model of depression.

Seizure testing: The anticonvulsant profile of Hit 8 was characterized in C57/B6, 129SV, Frings, and CF1 mice. The MES, 6 Hz, and corneal kindling seizure tests were conducted in C57/B6 and 129SV mice as described in Chapter 3. A genetic model of reflex epilepsy was also utilized and is

subsequently described, i.e., the Frings audiogenic seizure-susceptible mouse. In addition, CF1 mice were utilized in the subcutaneous pentylenetetrazol (scPTZ) test as described.

Audiogenic seizure test: The time to peak effect (TPE) for each compound was determined using 20 Frings mice; 4 at each of 5 time points (15, 30, 60, 120, and 240 min postinjection). The test compound was then administered at the TPE at varying doses (n=8 per dose, i.p.) until an ED_{50} could be obtained using a log-probit analysis. At the TPE, each mouse was tested for minimal motor impairment using the rotarod test as previously described (Dunham and Miya 1957; White et al. 2005). Immediately after the rotarod test, individual mice were then placed into a round Plexiglas jar (diameter 15 cm, height 18 cm, model AS-ZC, FET Research and Development, Salt Lake City, UT), where they were exposed to a sound stimulus of 11 kHz (110 decibels) for 20 sec. An animal was considered protected if it failed to display a full hindlimb tonic extension seizure. Mice were screened the day before drug testing to ensure that only responding animals were utilized.

s.c.PTZ test: The s.c.PTZ test detects the ability of a test compound to raise the seizure threshold of an animal and, thus, protect it from exhibiting a clonic seizure. At the previously determined TPE for the test substance, the dose of PTZ that will induce convulsions in 97% of mice (CD_{97}, 85 mg/kg, i.p.) is injected into a loose fold of skin in the midline of the neck. The animals are placed in isolation cages to minimize stress and observed for the next 30 min for the presence or absence of a seizure. An episode of clonic spasms, approximately 3

to 5 sec, of the fore and/or hindlimbs, jaws, or vibrissae is taken as the endpoint. The test compound was administered at the TPE at varying doses (n=8 per dose, i.p.) until an ED_{50} could be obtained using a log-probit analysis.

Formalin test: The test was performed using male C57/B6 mice as previously described (Murray et al. 1988; Tjolsen et al. 1992). The plantar region of the left hind paw of a mouse was injected with 5% formalin (20 µl with a 27 gauge stainless steel needle attached to a Hamilton syringe) 15 min (anticonvulsant TPE) following Hit 8 administration. Controls received a vehicle injection (0.5% methyl cellulose (MC), i.p.). Mice were acclimated in a 6" tall Plexiglas cylinder (4" diameter) for 15 min before receiving the formalin injection. Animals were observed for the first 2 min of each of nine 5-min epochs until 45 min had elapsed. The cumulative time spent licking in each 2-min period was recorded. Animals were immediately euthanized via CO_2 chamber following the test.

Forced swim test (FST): The FST was conducted in male C57/B6 mice as originally described (Porsolt et al. 1977). Testing was performed at the Hit 8 anticonvulsant TPE (i.e., 15 min). Results obtained from Hit 8 treated mice were compared to those obtained from vehicle-treated controls, which received a vehicle injection (0.5% methyl cellulose, i.p.). Mice were placed in 2000 ml glass beakers filled to a depth of 10 cm of clear water (24-25 ℃) and allowed one 5-min swim trial. The last 240 sec (4 min) of swimming was analyzed, as the first minute serves as habituation and induction of learned helplessness. A blinded reviewer recorded the time that was spent immobile with a stopwatch. A mouse

was considered immobile when it performed only enough movement to prevent submersion.

BGT1 specificity of Hit 8: Receptor binding profiles and K_i determinations for Hit 8 were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract # HHSN-271-2008-00025-C (NIMH PDSP). The NIMH PDSP is Directed by Bryan L. Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscol at NIMH, Bethesda MD, USA. For experimental details please refer to the PDSP web site http://pdsp.med.unc.edu/ and click on "Binding Assay" on the menu bar. Experiments were performed in quadruplicate.

Drug preparation: Hit 8 and TGB were suspended in 0.5% methyl cellulose (MC) (Sigma-Aldrich, St. Louis, MO) and sonicated for 15 minutes prior to administration to ensure complete dissolution or a micronized suspension. TGB was provided by Cephalon, Inc. (Frazer, PA), and Hit 8 was kindly provided by the laboratory of Rasmus P. Clausen, University of Copenhagen.

Statistical analysis: The anticonvulsant ED_{50} and 95% confidence interval (CI) for each compound was calculated using a log-probit analysis (Finney 1971a). Additional doses of each AED were administered until the 95% CI was inside the dose range evaluated, with a minimum of four doses administered. Formalin and FST data are presented as means + SEM. For the formalin test, mean area under the licking vs. time curve (AUC) was calculated for each group (GraphPad Prism, version 4.02 for Windows, GraphPad Software, San Diego, CA). A Student’s t-test was used to determine significant differences between groups in

the formalin test and FST. For all statistical comparisons, $p<0.05$ was considered significant.

Results

Anticonvulsant profile of Hit 8: The results obtained from a battery of well-defined seizure models using Hit 8 (15 min TPE) and TGB (1 h TPE) are summarized in Table 4.1. As shown in this table, Hit 8 displayed an anticonvulsant profile distinct from that of TGB. Both compounds were efficacious anticonvulsants at nontoxic doses in the Frings and corneal kindled mouse models. In contrast to TGB, Hit 8 was active in the MES model, whereas TGB was active in the 6 Hz and scPTZ models while Hit 8 was not (Table 4.1). These differential pharmacological profiles suggest that Hit 8 and TGB are working by different mechanisms of action (BGT1 and GAT1 inhibition, respectively).

Formalin test: Hit 8 was nonsedating at the dose utilized for the formalin test (20 mg/kg i.p.). A low dose was chosen to avoid sedation, which would confound the results of this behavioral test. Hit 8 was able to significantly reduce the pain response in both the acute and inflammatory phases of the formalin (Figure 4.1). In the acute phase, Hit 8 reduced paw licking to 35.82 ± 4.68% of control values ($p<0.01$). In the inflammatory phase, Hit 8 reduced paw licking to 51.25 ± 6.78% of control values ($p<0.01$).

Forced swim test: As shown in Figure 4.2, Hit 8 (30 mg/kg i.p.) significantly decreased the immobility time in the mouse FST ($p<0.01$). A decrease in

Table 4.1: Hit 8 and TGB anticonvulsant ED_{50} values (mg/kg, i.p.)

	Frings	**MES C57**	**MES 129**	**6 Hz C57**	**6 Hz 129**	**Kindling C57**	**Kindling 129**	**scPTZ CF1**
TGB	0.48 (0.38-0.66)	>10	>20	0.27 (0.16-0.41)	0.39 (0.23-0.66)	0.49 (0.29-0.71)	0.83 (0.65-1.03)	0.26 (0.14-0.45)
Hit 8	18.1 (15.6-20.7)	39.5 (32.1-43.1)	35.0 (29.7-40.5)	>70	>50	27.7 (20.0-38.7)	40.8 (32.9-48.8)	>100

ED_{50} values (mg/kg, i.p.) and (95% CI) for C57/B6, 129 SV or CF1 mice in various seizure models. C57 = C57/B6 mice; 129 = 129SV mice; CF1 = CF1 mice; Frings = Frings audiogenic seizure susceptible mouse model; MES = maximal electroshock model; 6 Hz = 6 Hz seizure model at 32 mA; Kindling = corneal kindling model; scPTZ = subcutaneous pentylenetetrazol test. >X = no higher doses were tested due to motor toxicity.

Figure 4.1: Analgesic action of Hit 8 in the formalin test. The licking response to formalin injection in the paw was investigated at 15 min following Hit 8 administration (20 mg/kg, i.p.). The AUC is reduced in the Hit 8-treated group compared to vehicle-treated controls in both phases of the test ($p<0.01$, $n=8$ per group).

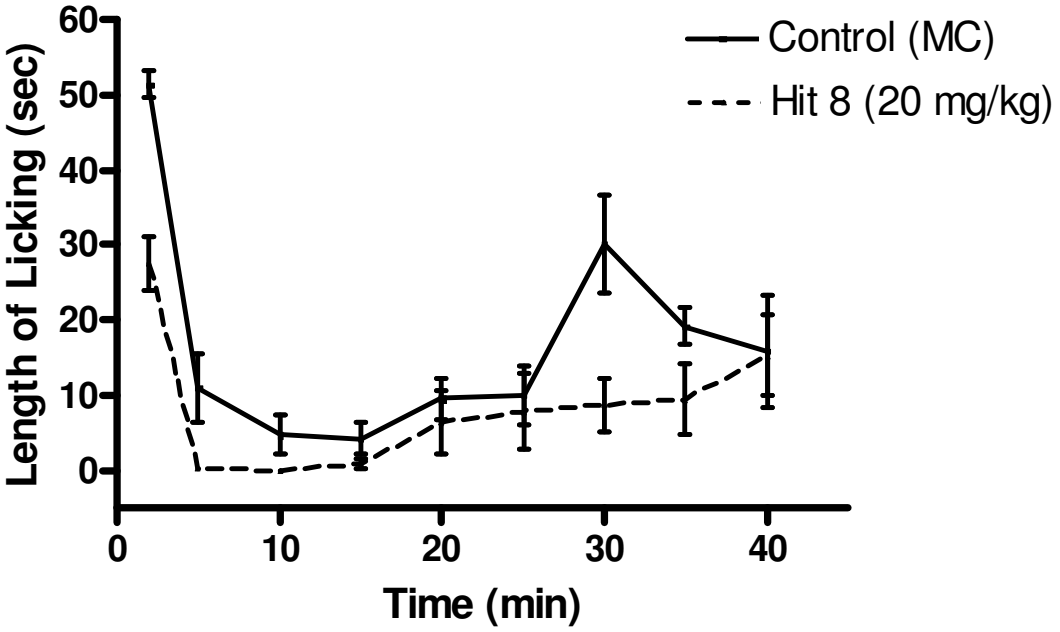
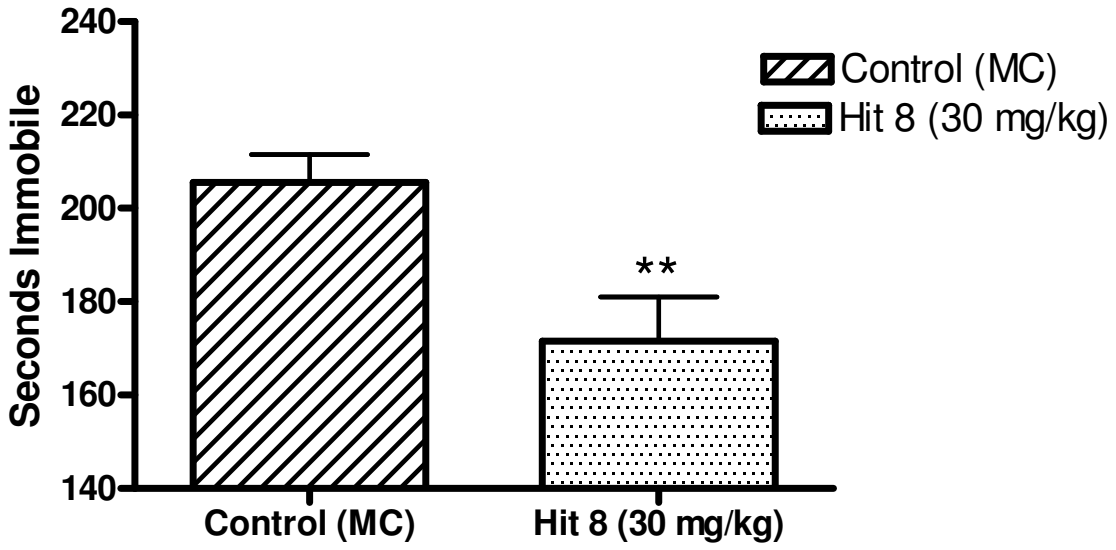

Control (MC)
Hit 8 (20 mg/kg)
Length of Licking (sec)
60
50
40
30
20
10
0
0
10
20
30
40
Time (min)

Figure 4.2: Hit 8 displays antidepressant activity in the FST. Animals were placed in a beaker of water (24-25℃) for 5 min. The last four minutes of the test was analyzed and the time that each animal spent immobile determined. $p<0.01$, n=8 per group.


240
220
200
180
160
140
Seconds Immobile
Control (MC)
Hit 8 (30 mg/kg)
**
Control (MC)
Hit 8 (30 mg/kg)

immobility induced by Hit 8 suggests that it may possess potential efficacy as an antidepressant.

BGT1 specificity of Hit 8: Hit 8 is specific for BGT1 of the GATs. However, further specificity testing was desired. Hit 8 was therefore submitted to the NIMH PDSP. Of the 47 receptor subtypes evaluated, Hit 8 displayed significant inhibition (>50%) of 13 receptor subtypes at 10 μM (results summarized in Table 4.2). These subtypes were further investigated and a K_i value determined, also shown in Table 4.2. The results from the NIMH PDSP reveal that Hit 8 is in fact not a BGT1-specific inhibitor.

Discussion

The screening of a small compound library identified Hit 8 as a possible selective BGT1 inhibitor. This method was utilized in order to identify an inhibitor that does not structurally mimic GABA, and so would be expected to bind outside of the relatively conserved binding pocket of the GATs and allow for the identification of GAT-specific inhibitors. Indeed, this screening method was able to successfully identify an inhibitor selective for BGT1 compared to the other GATs, a feat that proved difficult with structure-based approaches.

Hit 8 was found to possess anticonvulsant activity in the Frings and corneal kindling models and the MES test, but was inactive in the 6 Hz and scPTZ tests. Like Hit 8, TGB was also active in the Frings and corneal kindling models. In contrast, TGB was efficacious in the 6 Hz and scPTZ tests but not the MES test. TGB was also active in the Frings and corneal kindling models. Based on the

Table 4.2: Receptor subtypes significantly inhibited by Hit 8

Receptor subtype	% inhibition	K_i (nM)
5ht1a	49.2	8659
5ht1d	77	584
5ht2b	93.9	368
Alpha2C	74.1	648.7
D1	95.3	1260
D2	52.5	3118
D4	96.9	55
H1	85	560
M1	61.3	>10000
M3	55	>10000
M4	58.8	7971
M5	89.1	2886
MOR	51.4	3428

% inhibition and K_i values (nM) for Hit 8 at receptor subtypes as assayed by the NIMH PDSP. 5HT, serotonin receptor subtype; alpha, adrenergic receptor subtype; D, dopamine receptor subtype; H, histamine receptor subtype; M, muscarinic receptor subtype; MOR, mu opioid receptor.

differential pharmacological profile of Hit 8 compared to TGB, BGT1 inhibitors could theoretically be used to treat different seizure types than TGB, and the combination of these two drugs could be clinically useful to treat a broad spectrum of seizure disorders. Furthermore, BGT1 inhibition may potentially possess a differential and possibly less concerning adverse effect profile than TGB. Unfortunately, Hit 8 displayed behavioral toxicity and seizures at high doses. Hit 8 is analgesic, evidenced by its ability to reduce the pain response in both the acute and chronic phases of the formalin test at a dose devoid of toxicity. Hit 8 was also able to increase the active time in the forced swim test of depression, suggesting it may have potential antidepressant utility. The aforementioned data support the proposed function of Hit 8, e.g., BGT1 inhibition is expected to increase available extracellular GABA and be anticonvulsant, analgesic, and antidepressant. However, it remained unclear if BGT1 inhibition was indeed responsible for these results.

To address the functionality of BGT1 inhibition in the mechanism of action of Hit 8, the compound was screened for activity at other molecular targets. The primary binding results for Hit 8 indicated that it may not be BGT1 specific; Hit 8 significantly inhibited ligand binding to 13 of 47 targets screened. Hit 8 had K_i values under 10 μM at 11 of these targets. The affinity of Hit 8 for these targets compared to the affinity for BGT1 makes them of potential relevance in the *in vivo* testing that was performed, although the brain penetration of Hit 8 is unknown. Of particular interest is the fact that Hit 8 inhibits targets that are known to modulate seizures, depression, and pain. Several serotonin knockout animal

studies as well as human PET imaging studies support the hypothesis that serotonin plays a role in epilepsy (Theodore 2003). The selective serotonin reuptake inhibitor fluoxetine displays anticonvulsant activity at certain doses, and $5HT_{1A}$ stimulation is anticonvulsant in several experimental models of epilepsy (Wada et al. 1993; Gariboldi et al. 1996; Lu and Gean 1998; Hernandez et al. 2002). However, antagonism has also been reported to be anticonvulsant (Browning et al. 1997), and $5HT_{1A}$ agonists have been reported to increase spike-wave discharges (Filakovszky et al. 1999). $5HT_3$ stimulation has been suggested to be excitatory (Wada et al. 1997). Hence, serotonin receptor modulation could explain the anticonvulsant action of Hit 8 as well as its proconvulsant activity at high doses. Activity at dopamine receptors could also explain this effect: D1 stimulation is proconvulsant while D2 stimulation is anticonvulsant (Barone et al. 1991). It would be expected that inhibition of each target would produce the opposite effect. The opioid (la Fougere et al. 2009), histamine (Tuomisto et al. 2001), and muscarinic (Dupont et al. 1999) systems are also involved in epilepsy. Serotonin is a known modulator of depression, albeit via an unknown mechanism (Young et al. 1985; Delgado et al. 1990; Graeff et al. 1996). Modulation of the serotonin system may be functionally important in Hit 8's mechanism of action and be responsible for the results obtained in the FST. Hit 8 binds to subunits of serotonin receptors, mu opioid receptors, histamine receptors, dopamine receptors, and muscarinic receptors, all of which possess pain-modulating effects (Millan 2002). Therefore, many

targets of Hit 8 may play a role in producing the results observed in the formalin test.

With the currently available data, it seems likely that BGT1 inhibition is only a part of Hit 8's mechanism of action. It should be kept in mind that a lack of effect of BGT1 inhibition in the context of Hit 8 does not negate the possibility that a truly specific BGT1 inhibitor would be therapeutically useful. However, the inability of BGT1 to modulate neuronal excitability in the context of seizure control is further demonstrated in Chapter 5 by a lack of seizure phenotype in BGT1 KO mice. In the future, it would be of interest to administer Hit 8 to BGT1 KO and WT animals. If Hit 8 retains its effects in BGT1 KO animals, then BGT1 inhibition is not a major contributor to Hit 8's mechanism of action. As discussed in Chapter 5, the behavioral phenotype of BGT1 KO mice suggests that BGT1 remains a possible therapeutic target for pain and neurospsychiatric disorders, and the efforts to define specific BGT1-targeting compounds should therefore continue.

A screening technique was used to identify a compound that was not a GAT substrate or GABA analog but displayed GAT inhibitory activity selective for BGT1, not the other GATs. Although this approach was able to identify an inhibitor specific for BGT1 compared to the other GATs, it unfortunately led to the identification of a compound with several non-GABAergic targets. A specific BGT1 inhibitor therefore remains to be defined, and Hit 8 may potentially provide a lead structure. A BGT1-specifc inhibitor will be required to evaluate the potential clinical utility of this class of compounds.

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CHAPTER 5

BEHAVIORAL AND *IN VITRO* PHENOTYPE OF MICE LACKING THE BETAINE/GABA TRANSPORTER

Introduction

The betaine/GABA transporter (BGT1) is able to transport both the organic osmolyte betaine and the inhibitory neurotransmitter GABA (Lopez-Corcuera et al. 1992). GABA is a powerful modulator of CNS excitability, and has been implicated in several neurological disorders including epilepsy, pain, depression, anxiety, and other neuropsychiatric disorders (Koran 1976; Jasmin et al. 2004; Kalueff and Nutt 2007). The GABA hypothesis of epilepsy states that a decrease in GABAergic tone creates an imbalance between excitatory and inhibitory neurotransmission that can result in seizure activity. It has been previously demonstrated that pharmacologically increasing GABA leads to decreased seizure activity, while decreasing GABA is proconvulsant (Sinclair 1962; De Deyn et al. 1990; Dalby and Mody 2001). Several GABA-modulating drugs that were originally marketed as anticonvulsants are becoming increasingly utilized as pain medications. Tiagabine (TGB), pregabalin, gabapentin, and valproic acid are all prescribed for different varieties of pain and possess a mechanism of action involving GABA modulation (Todorov et al. 2005; Landmark 2007). Clinical data

also indicate efficacy of several GABAergic drugs including valproic acid, vigabatrin, gabapentin, and TGB for the treatment of depression, anxiety, and/or the manic phase of neurological disorders such as bipolar disorder (Kaufman 1998; Meldrum and Chapman 1999; Hosak and Libiger 2002; Vieta et al. 2006; Kalueff and Nutt 2007).

GABAergic signaling is terminated by reuptake of GABA into cells through GABA transporters (GATs) including GAT1, GAT2, GAT3, and BGT1. The specific GAT1 inhibitor, tiagabine (TGB), is a clinically utilized anticonvulsant with efficacy in pain and depression (Kalueff and Nutt 2007; Landmark 2007). Unfortunately, TGB is unable to ubiquitously produce seizure freedom and is proconvulsant under certain circumstances (Trinka et al. 1999; Balslev et al. 2000; Genton et al. 2001). Furthermore, although TGB is relatively well tolerated, its use is limited by pharmacokinetic factors and side effects including dizziness, fatigue, and confusion (Genton et al. 2001). A better-tolerated GABA uptake inhibitor is therefore desirable. *R,S*-EF1502 is a compound that has approximately equal affinity for GAT1 and BGT1 (Clausen et al. 2005). Co-administration of *R,S*-EF1502 with TGB resulted in a synergistic anticonvulsant effect in the absence of enhanced toxicity (White et al. 2005; Madsen et al. 2009). Furthermore, *R,S*-EF1502 decreased spontaneous interictal-like bursting frequency in brain slices bathed in hyperexcitable medium, while TGB failed to do so (Smith et al. 2008), suggesting that the BGT1 action of *R,S*-EF1502 is functional in its anticonvulsant mechanism. BGT1 inhibition may possess a therapeutic profile similar to TGB with a decreased incidence of side effects due

to differential localization, expression patterns, and affinity for GABA compared to GAT1. Unfortunately, there are no specific BGT1 inhibitors available to test this hypothesis. Therefore, a recently created BGT1 knockout mouse (Lehre et al, 2010, unpublished) has been utilized to determine the behavioral phenotype created by the lack of BGT1.

The current study was performed to determine the behavioral phenotype expressed by mice lacking BGT1 in order to assess whether BGT1 inhibition may be a useful therapeutic tool for the treatment of various CNS disorders. The behavioral profile of BGT1 KO mice was evaluated in a battery of animal models that evaluated their susceptibility to seizures, depression, anxiety, pain, and alterations in locomotion. Long-term potentiation (LTP) was investigated as well to determine the likelihood of cognitive side effects resulting from interference of BGT1 function. The results from this study support the further investigation of BGT1 modulation in neurological disorders.

Methods

Animal care: Male and female BGT1 KO and WT littermate controls (18-30 g) were utilized for seizure threshold studies, while only male mice were utilized for other behavioral testing. Animals were housed in a temperature-, humidity-, and light-controlled (12h light:dark cycle) facility. Mice were group-housed and permitted free access to food and water. All experimental procedures were performed in accordance with the guidelines established by the National

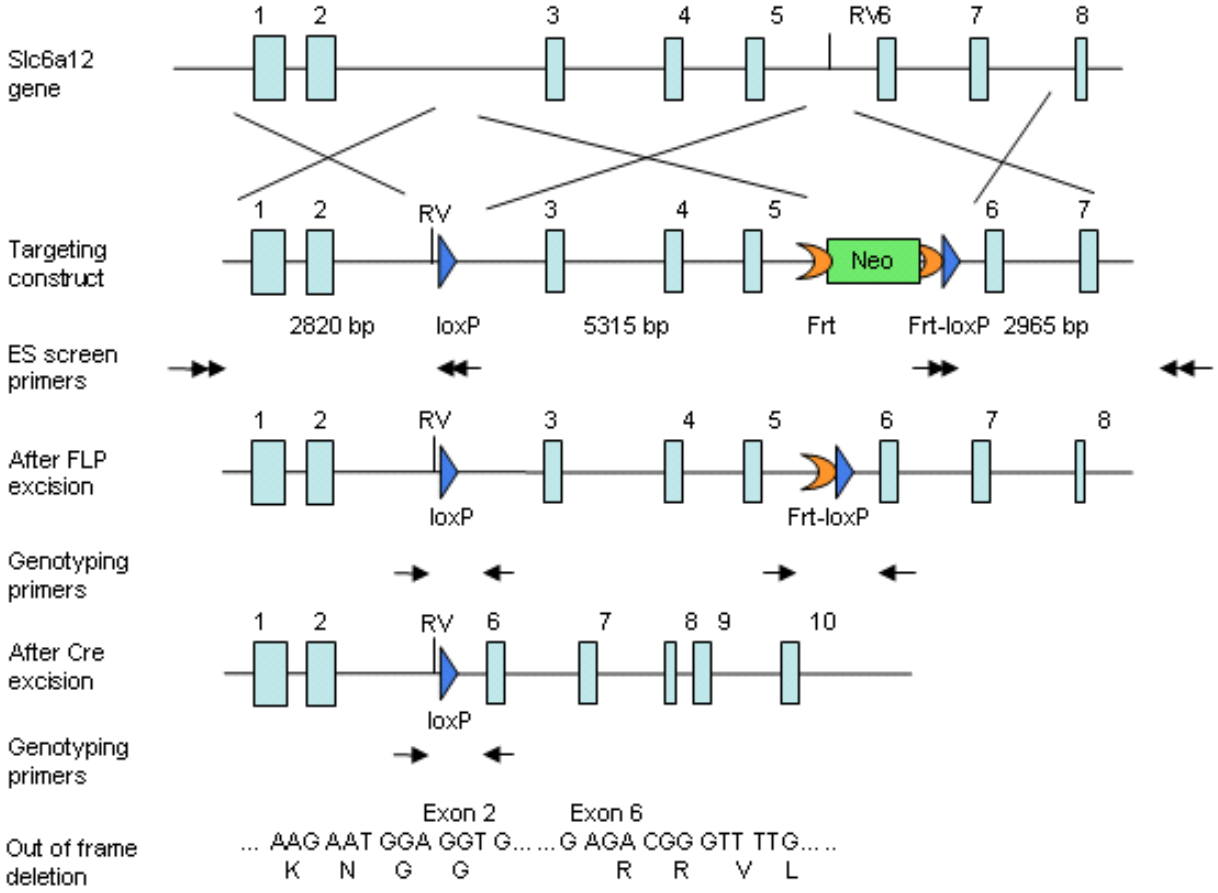
Institutes of Health (NIH) and received approval from the University of Utah's Animal Care and Use Committee (IACUC).

Breeding and genotyping of KO/WT mice: Heterozygous mice on a mixed C57/B6 and 129/SV background were obtained from Dr. Niels Christian Danbolt at the University of Oslo, Norway. Heterozygotes were bred, yielding KO, WT, and heterozygous offspring. The construct for BGT1 KO mice lacking exons 3-5 of BGT1 is shown in Figure 5.1. At the time of weaning, a tissue sample was obtained from each mouse and Proteinase K-digested at 65°C for 4.5 hours. Digestion was terminated by incubation at 80°C for 20 minutes and the samples were submitted to PCR. Primers utilized were: WT forward 5'-GGTGCATGCTTAAGACTCTG-3'; KO forward 5'-TGACCTCAAG CTGAATCCAC-3'; and both reverse 5'-CAAGCAGATATGGGAAGGCT-3'. Samples were run on a 2% agarose gel until clear band differentiation was possible. The wildtype allele appeared as a band at 147 bp, while the knockout appeared as a band at 203 bp. Heterozygotes displayed both bands. For all subsequent experiments, WT and KO littermates were compared.

Plasma osmolality of KO and WT mice: Blood was obtained from mice by decapitation and plasma separated by centrifuging for 3 min at >3,000 g. Plasma osmolality was determined utilizing a freezing-point depression osmometer (Advanced Instruments, Inc., Model 3320 Osmometer, Norwood, MA).

Compensation in the KO mice: mRNA levels of genes that may compensate for BGT1 deletion were investigated, including: GABA transporters 1 and 3

Figure 5.1: Generation of the BGT1-flox mice. The gene-targeting construct contained exon 1-7 of the BGT1 gene. A loxP sequence (blue arrow) was inserted into intron 2 and an EcoRV (RV) site was created at the front of the loxp site. A frt-PGKneo-frt-loxP cassette was inserted into intron 5 and an endogenous EcoRV (RV) site was deleted. The lengths of the homologous arms and the floxed fragment are indicated below the construct. The black arrows indicate the positions of the primers used for ES cell screening. The neo cassette was removed in the BGT1-flox mice generated from chimera x Rosa26FLP crossing. The genotyping primers are indicated by black arrows. After Cre excision the DNA encoding amino acid residues 72-193 are deleted, which causes the remaining sequences to be out of frame. Furthermore, the deleted region is essential for transport activity (Bismuth et al. 1997; Yamashita et al. 2005).


Slc6a12 gene
1
2
3
4
5
RV6
7
8
Targeting construct
RV
Neo
6
2820 bp
loxP
5315 bp
Frt
Frt-loxP
2965 bp
ES screen primers
After FLP excision
After Cre excision
Genotyping primers
Exon 2
Exon 6
Out of frame deletion
... AAG AAT GGA GGT G... ...G AGA CGG GTT TTG... ..
K N G G R R V L

(GAT1 and 3), the sodium-myoinositol co-transporter (SMIT), the taurine transporter (TauT), aldose reductase (AR), and tonicity-responsive enhancer binding protein (TonEBP). RNA was extracted and processed with RNeasy miniprep kits (QIAGEN, Valencia, CA) according to the manufacturer's instructions. A NanoDrop 3300 Spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA) was utilized to calculate RNA concentrations based on spectral absorbance at 260/280 nm. A standard RT reaction was run to produce cDNA using SuperScript II (Invitrogen, Carlsbad, CA). Osmolyte-related, GAT1 and GAT3 internal standards for PCR were cloned, while the standards and primers for BGT1 were purchased from Origene (Rockville, MD). The University of Utah DNA Sequencing Core Facility confirmed the cloned DNA sequences and a BLAST search was performed to confirm gene specificity. The quantitative PCR reaction contained 12.5 µl of RT^2 Real-Time SYBR Green PCR Master Mix (SA Biosciences, Frederick, MD), 10.5 µl of purified water, 1 µl of 10 µM primer set, and 1 µl of cDNA template. Quantitative PCR was performed using a PTC-200 Peltier Thermal Cycler with a Chromo4 Continuous Fluorescence Detector (MJ Research Inc., Waltham, MA). Data were analyzed with MJ Opticon Monitor Analysis Software, Version 3.00 (Bio-Rad Laboratories, Hercules, CA). All values were normalized to Proteosome subunit β 6 (Psmb6), a housekeeping gene with minimal variability (Rubie et al. 2005). Primer sequences, annealing temperatures, and product sizes are presented in Chapter 2. Each gene was amplified for 35 cycles.

Seizure threshold determination: Seizure threshold tests included acute electrical tests (minimal clonic, minimal tonic extension, and 6 Hz seizure threshold tests) and a chemical threshold test (intravenous pentylenetetrazol (i.v.PTZ)). In addition, the rate of corneal kindling acquisition was estimated using the corneal kindled mouse model of partial seizures. All of the electrical tests employed transcorneal stimulation with a Woodbury-Davenport stimulator (MES and corneal kindling tests, Woodbury and Davenport, 1952) or a Grass Stimulator (6 Hz test, Grass Technologies, West Warwick, RI). Different stimulation protocols (described by Otto et al., 2009) were utilized to differentiate the effects of BGT1 knockout on limbic (6 Hz partial seizures, 6 Hz, 3 sec), forebrain (minimal clonic seizures, 50 Hz, 0.2 sec), and hindbrain (minimal tonic extension seizures, 50 Hz, 0.2 sec) seizure thresholds. The CC_{50} (convulsive current necessary to produce the desired seizure endpoint in 50% of the population) was determined via the staircase procedure (Finney 1971b). Groups of 8 mice each were subjected to various stimulus intensities until at least four points were established between the intensity that failed to induce the desired seizure type and that which induced the desired seizure in 100% of mice. Additional stimulations were given as needed until the 95% confidence interval (CI) was inside the range of stimulations tested. The rate of corneal kindling acquisition was estimated according to the protocol described by Matagne and Klitgaard (1998) and as described in Chapter 3. The number of stimulations necessary to reach a fully kindled state (at least 5 consecutive stage 5 seizures, Racine 1972) was recorded for each mouse. For the i.v.PTZ test, a 0.5%

heparinized PTZ solution was infused at 0.24 ml/min into a lateral tail vein of an unrestrained mouse. The time to first twitch and to the onset of sustained clonus were recorded. The time to each endpoint was converted to the amount of PTZ infused (mg/kg) using the formula found in the analysis section.

Forced swim test: The test was conducted essentially as originally described (Porsolt et al. 1977). Mice were placed in 2000 ml beakers filled to a depth of 10 cm of water (24-25 ºC) and allowed one 5-minute swim trial. The last 240 sec (4 min) of swimming was analyzed, as the first minute serves as habituation and induction of learned helplessness. A blinded reviewer recorded the immobility time with a stopwatch. A mouse was considered immobile when it performed only enough movement to prevent submersion.

Light-dark box: Animals were placed in a locomotor detection chamber (AccuScan Instruments, Inc., Columbus OH) in a darkened room. Half of the chamber was brightly lit with an overhead lamp while the other half was a dark enclosure created by a black plexiglass box. Animals were placed in the lighted portion of the enclosure facing away from the entrance to the black box (4” x 1.25”). Activity was monitored for 10 min (600 sec), and the total time spent in the dark box was utilized to evaluate anxiety-like behavior in the mice.

Locomotion and mania tests: Animals were placed in a locomotor detection chamber (16.5” x 16.5” x 12”, AccuScan Instruments, Inc., Columbus OH) for 15 min prior to recording for habituation. Animals were recorded for 30 min to determine their basal locomotor activity. One week later, the same mice were injected with chlordiazepoxide (CDZP, 6.25 mg/kg, i.p.) and d-amphetamine (d-

AMPH, 1.25 mg/kg, i.p.) in an attempt to induce mania. Mice were recorded at the time-to-peak-effect (TPE) of CDZP and d-AMPH (30 min) following a 15 min habituation period. A separate group of mice were injected with lithium chloride (100 mg/kg, i.p.) and evaluated at the TPE (30 min) following a 15 min habituation period. Total distance traveled (cm) was used to evaluate the effect of BGT1 knockout.

Rotarod test*:* Mice were tested for motor deficits using the rotarod test (Dunham and Miya 1957). Briefly, mice were placed on a 1" diameter rod rotating at a speed of 6 revolutions/min. Inability to maintain equilibrium on the rotating rod was assessed. Latency to the first fall as well as total falls in 240 sec were recorded.

Tail-flick test: The tail-flick test was performed essentially as originally described (D'Amour and Smith 1941) with modifications using a tail-flick apparatus/analgesic meter (IITC Model 336 Plantar/Tail Analgesic Meter with True tail Temp and Heated Glass, IITC Life Science Inc., Woodland Hills, CA). The cutoff latency was set at 25 sec to avoid tissue damage. Mice were habituated to the restraining device for 3 min approximately 4 hours prior to the time of the test, and again for 1 min immediately prior to the test.

Writhing test: Acetic acid (0.6% v/v and 1.2% v/v, 10 mg/kg) was injected into the peritoneal cavities of mice. Mice were immediately observed for 30 min in observation chambers and the number of writhes counted (Collier et al. 1968). Writhing is characterized by the contraction of abdominal muscles with concurrent hind limb stretching. Animals were subsequently euthanized with CO_2.

Formalin test: The formalin test was performed as described previously (Murray et al. 1988; Tjolsen et al. 1992). Corneal kindled mice were acclimated in a 6" tall Plexiglas cylinder (4" diameter) for 15 min before receiving a 5 % formalin injection (20 µl with a 27 gauge stainless steel needle attached to a Hamilton syringe) in the plantar region of the left hind paw. Animals were observed for the first 2 min of nine 5-min epochs until 45 min had elapsed. The cumulative time spent licking in each 2-min period was recorded. Kindled mice were utilized due to the unavailability of naïve WT and KO males. Animals were subsequently euthanized with CO_2.

Carrageenan-induced thermal hyperalgesia: A state of localized inflammation was induced in mice by injecting carrageenan (25 µl, 2% in 0.9% NaCl, λ-carrageenan, Sigma-Aldrich, St. Louise, MO) subcutaneously into the plantar surface of the right hind paw (Kerr et al. 2001; Gonzalez-Rodriguez et al. 2010). Paw withdrawal responses from thermal stimulation were assessed according to previously described methods (Hargreaves et al. 1988; Dirig et al. 1997; Hua et al. 2005). Mice were placed in plexiglass chambers on top of a heated glass surface maintained electronically at a constant temperature (here, 30 ℃). Thermal stimulation was applied with a projection bulb below the glass surface. Latency to paw withdrawal was measured from the onset of heat application until a full paw withdrawal occurred. After a habituation period, baseline withdrawal latencies were assessed at -30 min. Carrageenan was injected at time 0, and withdrawal latencies were thereafter assessed every 30 min (30-210 min). At each time point, measurements were taken from each paw (injected, ipsilateral

and non-injected, contralateral), with at least 1 min between measurements. Two measurements were obtained from each paw and subsequently averaged to obtain the mean withdrawal latency for each paw at each time point. Experimental conditions, including animal habituation, glass plate temperature, and thermal stimulus intensity have been optimized such that baseline withdrawal latencies for contralateral and ipsilateral paws were 6-9 sec and 2-4 sec, respectively (Dr. Cameron Metcalf, unpublished data). After the final time point tested, paw edema was measured using a caliper. Animals were subsequently euthanized with CO_2.

Hippocampal brain slice preparation: The preparation of brain slices and LTP experiments were designed, conducted, and analyzed by Dr. Peter West. Male BGT1 WT and KO mice were sacrificed by decapitation and brains were rapidly removed and placed in ice cold (4°C) oxygenated artificial cerebral spinal fluid (ACSF) (95% O_2/5% CO_2) containing (in mM): NaCl (126.0), KCl (3.0), Na_2PO_4 (1.4), $MgSO_4$ (1.0), $NaHCO_3$ (26.0), glucose (10.0), and $CaCl_2$ (2.5) – pH (7.25-7.35) and osmolality (290-300 mOsm). Brains were then blocked and glued to the stage of a vibrating blade microtome (VT1000S, Leica Microsystems, Inc., Bannockburn, IL). Coronal brain slices (400 µm) containing the dorsal hippocampus were cut and then incubated in oxygenated ACSF at room temperature for 1-2 h prior to recording. The oxygenated ACSF used for this procedure is the same as that used for slice incubation and recording, and the pH and osmolality of this solution were verified prior to each experiment.

LTP at the Schaffer collateral-commissural projection to CA1 pyramidal neurons: Extracellular field potentials were recorded using a Slicemaster high throughput semiautomated brain slice recording system (Scientifica, UK). This system permits concurrent and independent recordings from four to eight brain slices, thus improving throughput, statistical design, and reducing animal use (Stopps et al. 2004). Slices were transferred to the integrated brain slice chambers (IBSCs) of this system, were held in place between two nylon nets, and were continuously perfused with oxygenated ACSF at a flow rate of 2.5 mL/min. Recordings were performed at 30-31°C. Field potentials from four independent brain slices were recorded simultaneously. The total n represents brain slices from animals of each genotype.

Concentric bipolar stimulating electrodes (MCE-100, Rhodes Medical Instruments, Summerland, CA) were placed in the stratum radiatum of CA1 in order to stimulate the Schaffer collateral-commissural axons and evoke field excitatory post-synaptic potentials (fEPSPs). Recording microelectrodes pulled from borosilicate glass (1.5 mm O.D., 1.12 mm I.D) using a PIP5 pipette puller (HEKA, Bellmore, NY) were filled with ACSF and placed within 300-500 µm of the stimulating electrodes in the stratum radiatum of CA1. Data were acquired using custom software written in Spike2 (CED, Cambridge, England) at a sampling rate of 10 kHz and low-pass filtered at 1 kHz. One hundred µs stimuli ranging from 5-50 V were used to evoke fEPSPs, and the magnitude of the fEPSP was determined by measuring the 20-80% slope of the rising phase. Input-output curves were generated for all slices and the baseline stimulation

strength was set to 50% of the range between the minimum and maximum fEPSP. Slices were stimulated every 30 s for 30 min at which point the degree of LTP was determined relative to baseline.

Analysis: Data are presented as means ± standard error. For the i.v.PTZ test, the amount of PTZ infused (mg/kg) was calculated using the formula: (19.95 x infusion time (sec) (T)) / weight of animal in grams (W). This formula is derived from the calculation of mg/kg PTZ = (T x rate of infusion (ml/min) x mg PTZ/ml x 1000g) / (60 sec x W in grams) = (T x 0.2394 x 5 x 1000) / 60 x W = (19.95 x T) / W. For mRNA compensation studies, the copy number of a specific gene in a sample was normalized to the value of the housekeeping gene, Psmb6, for that sample. Results are expressed relative to a mean control value of 100%, i.e., each experimental group was normalized to a mean control value of 100% by dividing each experimental value by the average control value. For the carrageenan test, mean withdrawal latencies for contralateral and ipsilateral paws were plotted separately for each group (WT and KO) as a function of time. Comparisons between multiple means were performed using a one-way repeated measures ANOVA followed by Tukey's multiple comparisons test. For the formalin test, mean area under the licking v. time curve was calculated for each group. For LTP experiments, data were excluded if any of the following were observed: erratic changes in the slope of fEPSPs during the 30 min baseline recording, lack of a clear and concise increase in the slope of the fEPSP immediately after TBS (no post-tetanic potentiation), or any sudden erratic changes in the slope of the fEPSP post-TBS. Unless otherwise mentioned, a

two-tailed Student's t-test was used to determine if significant differences existed between KO and WT groups. $P<0.05$ was considered significant.

Results

Seizure threshold determination: Seizure thresholds were not different between WT and KO animals in the minimal clonic, 6 Hz, or minimal tonic extension tests, or in the i.v.PTZ threshold test. CC_{50} values (95% CI) for electrical tests and dose (mg/kg) of PTZ required to induce sustained clonus (means ± SEM) are shown in Table 5.1.

Compensation in KO mice: mRNA levels of genes that may compensate for BGT1 deletion were investigated, including GAT1 and 3, SMIT, TauT, AR, and TonEBP. None of the investigated genes were upregulated to compensate for the deletion of BGT1 (data not shown).

Plasma osmolality of KO and WT animals: Resting plasma osmolality values were determined to be 308.2 ± 1.7 mOsm/kg for WT mice (n=9) and 308.9 ± 1.3 mOsm/kg for KO mice (n=8). No differences in plasma osmolality existed between genotypes.

Porsolt forced swim test (FST): BGT1 KO animals (n=10) spent significantly greater time immobile than WT animals (n=8), shown in Figure 5.2. Therefore, KO mice appear to demonstrate a depressive-like phenotype in the FST.

Light-dark box: WT and KO mice (n=9 per group) were tested for anxiety utilizing the light-dark box. As seen in Figure 5.3, KO mice spent a significantly

Table 5.1: Seizure thresholds of BGT1 WT and KO mice (mA or mg/kg)

	WT	KO
Minimal clonic (mA)	8.6 (7.8-9.6)	9.0 (8.2-9.7)
6 Hz (mA)	30.4 (26.2-33.8)	31.2 (27.1-34.8)
Minimal tonic extension (mA)	13.1 (11.7-14.3)	13.7 (12.7-14.5)
i.v.PTZ (mg/kg to clonus)	44.5 ± 3.0	42.3 ± 1.5

Electrical seizure thresholds (minimal clonic, 6 Hz, and minimal tonic extension) were determined for WT and KO mice using the staircase method. CC_{50} values and (95% CI) are shown in milliamps (mA). Seizure threshold was also determined using the i.v.PTZ test. The dose (mg/kg, i.v.) of PTZ required to produce sustained clonus is reported (mean ± SEM). No differences exist between WT and KO animals in any of the seizure threshold tests performed.

Figure 5.2: BGT1 KO mice display a depressive-like phenotype in the FST. BGT1 WT (n=8) and KO mice (n=10) were tested using the Porsolt FST. KO mice spent more time immobile (means ± SEM) compared to WT mice in a 240 sec swim trial. $p<0.01$.

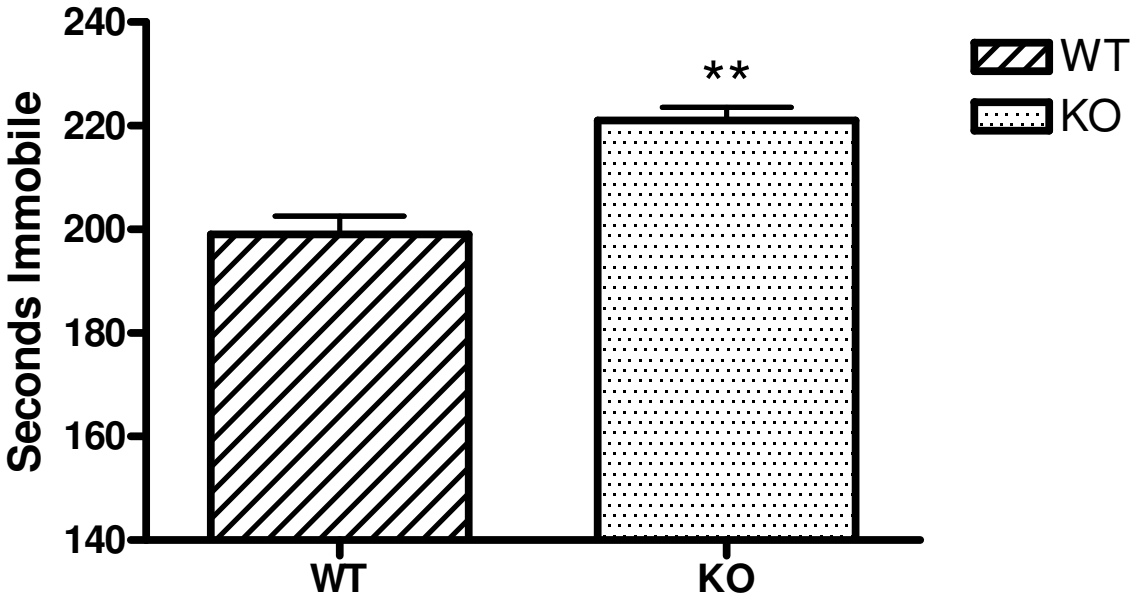
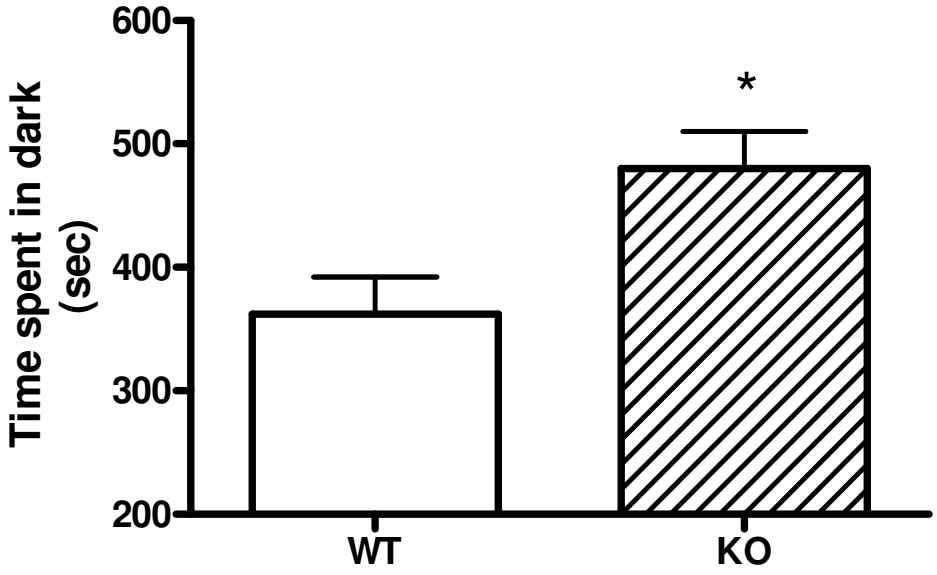

240
220
200
180
160
140
Seconds Immobile
**
WT
KO
WT
KO

Figure 5.3: BGT1 KO mice display an anxiety-like phenotype in the light-dark box. BGT1 WT and KO mice (n=9 per group) were tested for anxiety-like behavior using the light-dark box. KO mice spent more time in the dark portion of the test area (means ± SEM) compared to WT mice in a 600 sec trial. $p<0.05$.


600
500
400
300
200
Time spent in dark (sec)
*
WT
KO

greater amount of time in the dark portion of the box compared to WT mice. The results of this test suggest that BGT1 KO mice possess an anxiety-like phenotype in the light-dark box.

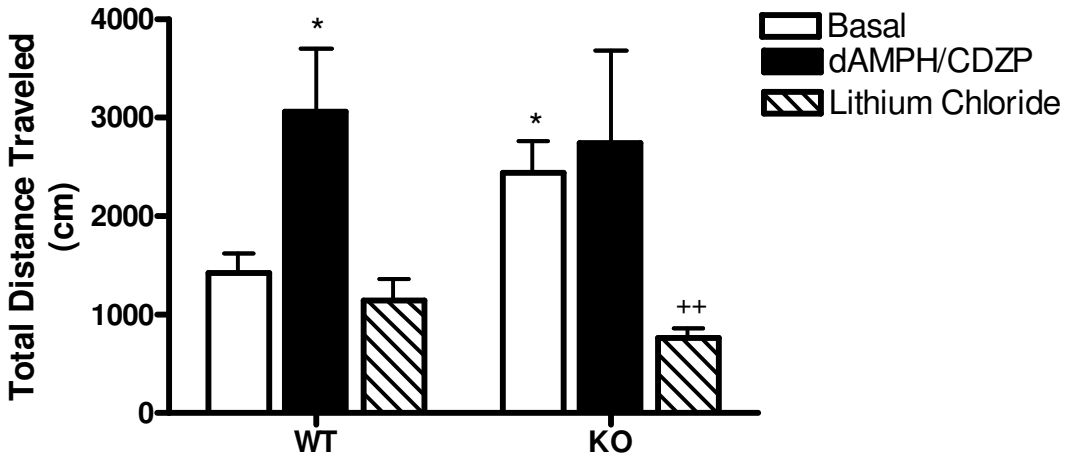
Locomotor profile following CDZP/dAMPH and lithium injection: As shown in Figure 5.4, BGT1 KO mice (n=16) have significantly increased basal locomotor activity compared to WT mice (n=15). However, hyperlocomotion was not increased further in the KO mice with CDZP/dAMPH administration (n=13). In contrast, this treatment significantly increased activity in WT mice (n=11). Administration of lithium (100 mg/kg) attenuated basal locomotion in KO mice, but not WT mice (n=8 per group). WT and KO mice traveled a similar distance following lithium administration. Hence, the BGT1 KO mouse has increased basal locomotion that is not increased by CDZP/dAMPH, but that is attenuated by lithium at a dose that has no effect on the basal locomotion of WT animals.

Rotarod test: BGT1 KO mice did not display motor impairment in the rotarod test ($p=0.33$, n=8 per group). No WT mice fell during the 4 min rotarod trial, and one KO mouse fell a single time at 21 sec.

Tail-flick test: No difference existed in the latency to tail flick between WT (2.37 ± 0.29 sec, n=11) and KO (2.33 ± 0.31 sec, n=9) mice (data not shown).

Writhing test: No difference was determined between WT (32.9 ± 3.6, n=11) and KO (37.2 ± 5.8, n=9) mice in number of writhes in 30 min induced by 0.6% acetic acid injection. Increasing the acetic acid concentration to 1.2% did not produce a difference between genotypes; WT (47.6 ± 6.1, n=10) and KO (53.9 ± 8.5, n=9).

Figure 5.4: BGT1 KO mice have high basal locomotion that is attenuated by lithium. Locomotion was determined in BGT1 WT and KO mice and expressed as total distance traveled (cm) in 30 minutes. Locomotion was significantly increased by CDZP/dAMPH injection (6.25 and 1.25 mg/kg, i.p., respectively) in WT but not KO mice. Basal locomotion was attenuated by lithium chloride (100 mg/kg, i.p.) in only KO mice. * $p<0.05$ v WT basal; ** $p<0.01$ v WT basal; ++ $p<0.01$ v KO basal.


Total Distance Traveled (cm)
4000
3000
2000
1000
0
*
*
++
WT
KO
Basal
dAMPH/CDZP
Lithium Chloride

Formalin test: As shown in Figure 5.5, BGT1 KO mice had a significantly decreased area under the curve (AUC) in the inflammatory phase (phase II) of the biphasic licking response test compared to WT mice (n=8 per group), suggesting an attenuated response to inflammatory pain. No significant differences were observed in the acute phase of the response (phase I).

Carrageenan test: As shown in Figure 5.6, there was no difference between genotype in carrageenan-induced thermal hyperalgesia. KO and WT groups exhibited significant hyperalgesia in the carrageenan-injected paw (n=8 per group, $p<0.05$), and displayed similar paw edema (KO, 2.8 ± 1.9 mm; WT, 2.7 ± 2.0 mm).

Long-term potentiation (LTP): As shown in Figure 5.7, there was no difference in LTP between BGT1 WT and KO mice. Although this result does not support the development of a BGT1 inhibitor for cognitive enhancement, it does provide evidence that BGT1 inhibition may likely be devoid of cognitive side effects.

Discussion

It was expected that BGT1 KO mice would have more available extracellular GABA and hence have an increased seizure threshold. However, no difference was observed in several different seizure threshold tests between genotypes, in agreement with what has previously been reported (Lehre et al. 2010, unpublished). However, in contrast with previous reports, there was a trend toward a difference in the time required to reach a fully kindled state in the corneal kindling model. This result is encouraging because corneal kindling is the

Figure 5.5: Corneal kindled BGT1 KO mice display an analgesic phenotype in the formalin test. Time spent licking the paw was determined following formalin injection. Results are represented as (mean ± SEM) and significance determined by a Student's t-test of total AUC. The time spent licking the injected paw was significantly less in the KO mice compared to WT mice ($p<0.05$, $n=8$ per group).

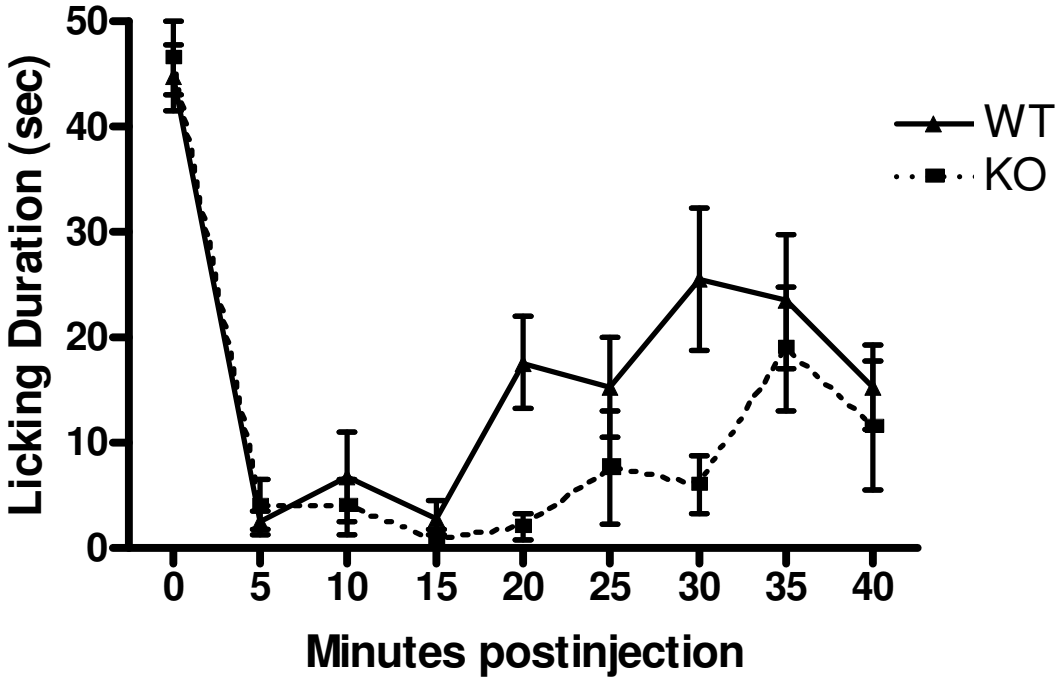
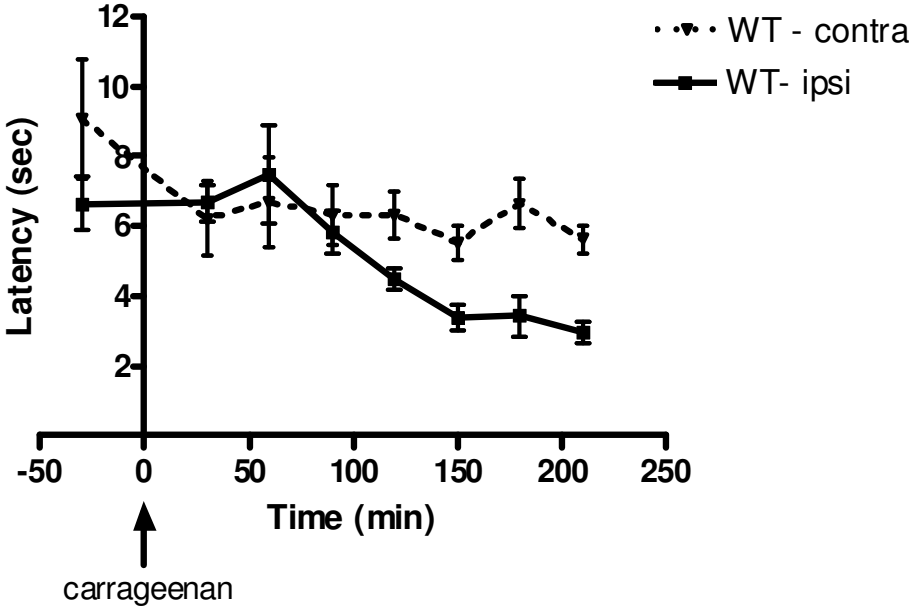

Licking Duration (sec)
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
Minutes postinjection
WT
KO

Figure 5.6: BGT1 WT (A) and KO (B) mice exhibit a similar response to carrageenan-induced thermal hyperalgesia (ipsi/contra $p>0.05$). Latency (sec) to paw withdrawal was determined at several time-points following carrageenan injection. Both WT and KO mice withdrew the injected (ipsi) paw significantly faster than the non-injected (contra) paw ($p<0.05$, one-way repeated measures ANOVA with Tukey's multiple comparison's test). Results are represented as (means ± SEM).


WT - contra
WT- ipsi
Latency (sec)
12
10
8
6
4
2
-50
0
50
100
150
200
250
Time (min)
carrageenan

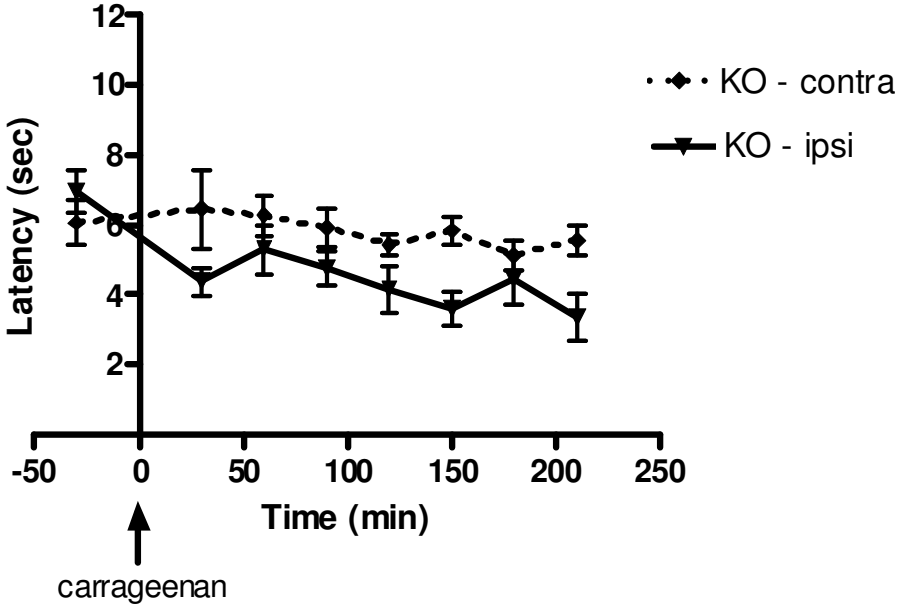
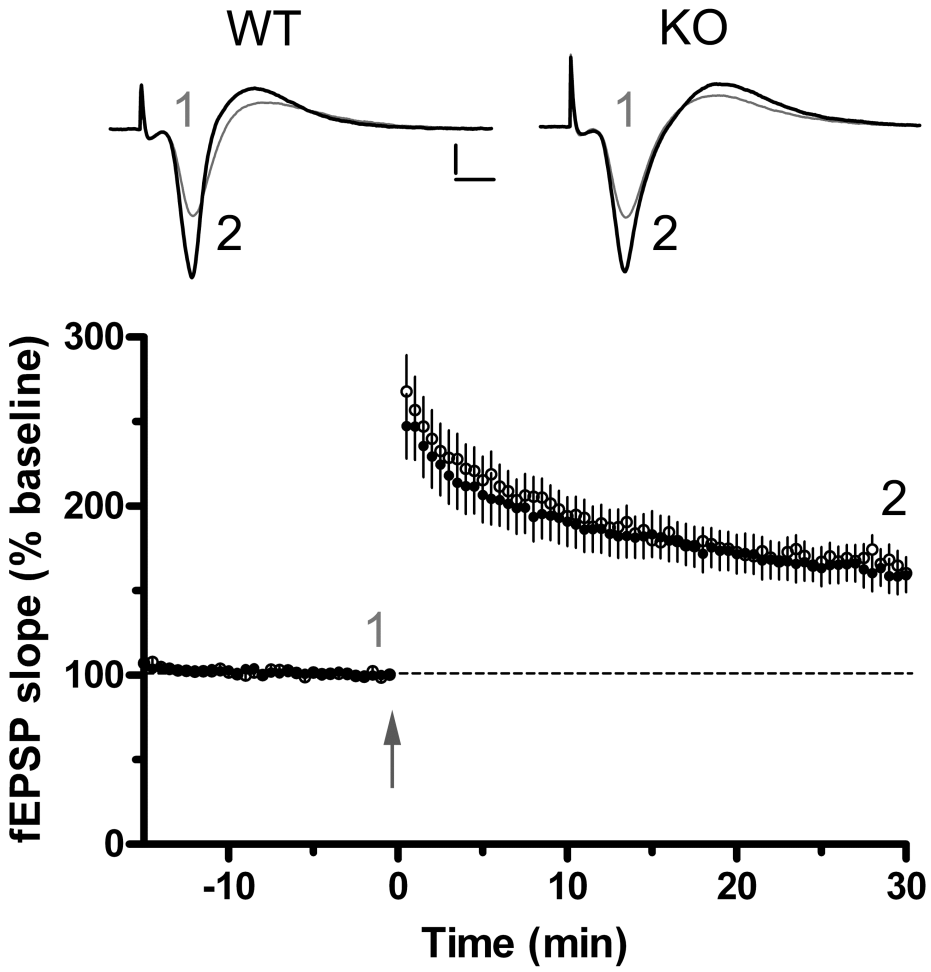

KO - contra
KO - ipsi
Latency (sec)
12
10
8
6
4
2
-50
0
50
100
150
200
250
Time (min)
carrageenan

Figure 5.7: LTP of excitatory synaptic transmission in the CA1 field of the hippocampus is normal in BGT1 KO mice. Representative traces of fEPSPs from hippocampal brain slices prepared from both WT and BGT1 KO mice are the average of 10 sweeps taken immediately before (1, gray) or 30 min after (2, black) TBS. Scale bars represent 0.25 mV and 5 msec. Lower panel: Single stimuli were given every 30 sec. fEPSP slope measurements made from either WT slices (○, N=20) or BGT1 KO slices (●, N=21) are represented as mean ± SEM. TBS (indicated by the arrow) induces a long lasting increase in the slope of the fEPSP.


WT
KO
1
2
1
2
300
200
100
0
fEPSP slope (% baseline)
2
1
-10
0
10
20
30
Time (min)

only long-term model utilized in the present investigation, and epilepsy is a chronic disease. It is possible that deletion of BGT1 activity has no effect on acute seizure threshold, but rather modulates the condition in a chronic capacity by modifying long-term inhibitory tone. BGT1 expression is altered in a model of epilepsy (Rowley et al. submitted, see Chapter 2), which supports the idea of chronic involvement of BGT1 in this neurological disorder.

One possible explanation for the lack of effect on seizure threshold is that developmental compensation may exist in the KOs. In the present study, other transporters (GAT1, GAT3, TauT, SMIT), a metabolic enzyme (AR), and a transcription factor (TonEBP) of interest were investigated and no compensation for BGT1 knockout was found. However, other compensatory mechanisms that were not investigated may be present. For example, GAT3 is not upregulated in the GAT1 KO mouse; however, GAD65/67 is increased in the cerebral cortex (Bragina et al. 2008), perhaps to enhance GABA synthesis to overcome the lack of re-uptake. Alterations in release characteristics and $GABA_B$ receptors have also been shown in GAT1 KO mice (Jensen et al. 2003; Xu et al. 2007).

The animals utilized in the current studies are conventional KOs and lack BGT1 in all tissues. Since BGT1 has a function to regulate osmotic balance in the liver and kidney, plasma osmolality of KO and WT animals was determined to ensure no systemic osmotic differences existed between genotype. Plasma osmolality is important because osmotic balance contributes to neuronal excitability (Schwartzkroin et al. 1998). However, no difference between

genotypes was observed regarding plasma osmolality, in agreement with the lack of notable hyperexcitability in acute seizure tests.

It has been suggested that BGT1 may not function to remove presynaptically released GABA due to its expression levels, localization, and affinity (Borden 1996; Zhu and Ong 2004). The current study provides *in vivo* evidence that BGT1 is not likely to be functionally responsible for controlling neuronal excitability in the context of seizure thresholds. Therefore, BGT1 is likely serving a function not involving the removal of presynaptically released GABA. BGT1 transports the osmolyte betaine in addition to GABA (Lopez-Corcuera et al. 1992). Osmotic regulation is an important aspect of brain homeostasis, and the ability to regulate osmolyte balance may confer functional significance to BGT1. In addition, it has been reported that BGT1 is the only GABA transporter present at the BBB (Takanaga et al. 2001), and that GABA is actively transported from the brain interstitial fluid to the blood through the BBB (Zhang et al. 1999; Kakee et al. 2001). Hence, BGT1 is the most likely candidate for mediating the efflux of GABA from the brain. BBB efflux does not impart an unprecedented function, and may pertain to betaine as well, because excitatory acidic amino acids and the osmolyte taurine are also effluxed across the BBB (Tamai et al. 1995; Hosoya et al. 1999). Another possible function for BGT1 is the redistribution of GABA from GABAergic to glutamatergic neurons (Sunol et al. 2010). Furthermore, BGT1 may function to redistribute betaine following osmotic stress, as has been shown to occur with taurine (Nagelhus et al. 1993). Since neurotransmitter transport is coupled to the influx of ions, a small current is generated upon uptake. However,

a much larger current can be generated via cation transport through GAT1, which is mediated by the activation of protein kinase A. This GAT1 current is generated without an increase in extracellular GABA, and suggests an additional function of GAT1 as a "neurotransmitter-gated ion channel" (Bagley et al. 2005). The excitatory amino acid transporters EAAT4 and EAAT5 are also known to function in a similar manner under certain conditions (Fairman et al. 1995; Arriza et al. 1997; Wersinger et al. 2006). Hence, several potential functions of BGT1 in the CNS remain to be investigated.

The BGT1 KO mouse displayed increased immobility in the FST and increased time spent in the dark portion of the light-dark box compared to WT animals. The result in the FST was not due to general hypoactivity of KO mice. On the contrary, BGT1 KO mice demonstrated significantly increased basal locomotor activity compared to WT mice in a locomotion detection chamber. The presence of a depressive-like phenotype in the FST and an anxiety-like phenotype in the light-dark test suggests that BGT1 may be functionally modulating the GABA system in the CNS, and that an inhibitor may possess neuropsychiatric side effects.

The BGT1 KO mouse has significantly increased basal locomotion compared to WT mice. Hyperlocomotion is a standard parameter examined in studies of neuropsychological disorders that involve a 'manic' phase, such as schizophrenia and bipolar disorder. Interestingly, BGT1 KO mice were resistant to pharmacologically-induced hyperlocomotion; although their basal locomotion was significantly increased compared to WT, CDZP/dAMPH did not further increase

the distance traveled. The basal and CDZP/dAMPH-induced movement of the KO mouse does not represent a “ceiling effect,” as mice that were not habituated walked up to 21,000 cm (approximately 5 times the distance traveled by habituated, CDZP/dAMPH-injected mice). Encouragingly, the administration of lithium to BGT1 KO animals significantly reduced their locomotion at a dose that was ineffective at decreasing basal locomotion in WT mice. The lithium-treated KO group traveled a similar distance to the lithium-treated WT group, and neither lithium-treated group traveled a significantly different distance than the naïve WT group. Therefore, the hyperlocomotor response of the BGT1 KO mouse is completely reversed by lithium at a dose devoid of locomotor activity in WT mice. This dose of lithium would be expected to not alter basal locomotion, but to significantly attenuate dAMPH -induced locomotion (Gould et al. 2007). The lack of effect of 100 mg/kg lithium in WT mice and the marked response of the KOs may suggest that the BGT1 KO mouse represents a genetic model of mania that is attenuated by lithium at doses that do not affect basal locomotion. In the future, it will be imperative to test known antipsychotic compounds in the KO mice to determine if indeed the BGT1 KO mouse produces results that are predictive of clinical efficacy. Taken altogether, the increased immobility observed in the Porsolt FST, the basal hyperlocomotion in the activity monitor, and the clear reduction in basal hyperlocomotion in response to LiCl administration would suggest that the BGT1 KO may represent a genetic model of bipolar disorder. This hypothesis may warrant further investigation.

Due to the increased immobility in the FST, the increased time in the dark portion of the light-dark box, and increased basal locomotion of the BGT1 KO mouse, it may be hypothesized that inhibition of BGT1 would produce unwanted side effects. The current study suggests that the development of a BGT1 agonist may be beneficial in neuropsychiatric disorders as opposed to an inhibitor. Since all of the preclinical models evaluated suggest that BGT1 does not directly affect seizure threshold, an agonist would not be expected to increase seizure susceptibility. However, this hypothesis would require further investigation to confirm the safety and utility of such an approach *in vivo*.

In the behavioral characterization of the BGT1 KO mouse, it was found that the KO mouse is less susceptible to pain induced by formalin injection, but no difference was observed in carrageenan-induced hyperalgesia or in paw edema following carrageenan injection. Two likely explanations exist for this observation. First, although both tests are models of inflammatory pain, they do produce hyperalgesia by differing mechanisms. Formalin activates a member of the Transient Receptor Potential family of cation channels, the TRPA1 cation channel, to induce hyperalgesia (McNamara et al. 2007), while a specific receptor has not been identified in the carrageenan-induced response. Hyperalgesia develops more slowly in the carrageenan test and involves a phenomenon termed “windup,” in which the number of neuronal responses progressively increases (Mendell 1966). Windup is involved in the maintenance of inflammatory and neuropathic pain and is attributed to responses of spinal dorsal horn neurons evoked by C-fiber afterdischarge (Dubner 1986; Gracely et

al. 1992). In contrast, the formalin test produces a rapid pain response that, at the acute times investigated, precedes the windup phenomenon. Second, the formalin test was performed in kindled mice while the carrageenan test was performed in naïve mice. It is a distinct possibility that the process of kindling differentially affected the pain threshold of WT and KO mice, and that, under these particular conditions, a difference in inflammatory pain exists between genotypes. This may be the most likely explanation for the contradictory results obtained. It was expected that, if a pain phenotype existed, it would be determined by the writhing test, which is the most sensitive pain test (Hendershot and Forsaith 1959; Collier et al. 1968). However, the writhing test did not determine a difference between genotypes. The results obtained in pain assays were unexpected, and may be a result of genetic variability as discussed below. Further investigation is necessary to determine what role BGT1 plays in the modulation of pain. In the future, the formalin test must be performed in naïve animals and paw edema measured. It may also be of interest to investigate the role of corneal kindling in pain modulation if no difference between genotypes is found in naïve mice.

It must be kept in mind when interpreting data obtained with genetically altered mice that WT and KO mice are not coisogenic, meaning they do not differ only in the targeted gene. Rather, genes that are on the same chromosome as the transgene are linked to the transgene and become hitchhiking donor genes (Crusio 1996). Mice for the current study were bred on a mixed 129/SV and C57/B6 background. Hitchhiking donor genes will be 129/SV-derived in KO mice

(since the transgene was derived from 129/SV embryonic stem cells) and C57/B6-derived in WT mice. Even after 12 generations of breeding, up to 300 hitchhiking genes may remain. Hence, phenotypic differences observed in KO mice may actually be due to differential alleles in these neighboring genes between mouse strains (Gerlai 1996). Hitchhiking genes are particularly confounding in pain studies, because 1) 129/SV and C57/B6 mice differ significantly in pain responses, and 2) C57/B6 mice are a poor representative species for pain studies, as they are clear outliers regarding many pain-related traits (Mogil et al. 2001). The existence of possible strain differences does not explain the lack of effect of dAMPH on BGT1 KO mouse locomotion, because both C57/B6 mice and 129/SV mice have been reported to experience significant hyperlocomotion in response to dAMPH (Gould et al. 2007). Strain differences also do not explain the increased basal locomotion of KO mice, because 129/SV mice are less mobile under basal conditions than C57/B6 mice. dAMPH/CDZP hyperlocomotion in 129 mice is not attenuated by 100 mg/kg lithium, while it is significantly reduced in C57/B6 mice (Gould et al. 2007). These results are not consistent with the observation that basal locomotion of BGT1 KO mice (more 129/SV genes vs WT mice) is significantly attenuated by 100 mg/kg lithium while WT locomotion is not. In the future, the combination of dAMPH/CDZP and lithium must be determined in WT and KO mice as performed by Gould et al. (2007). In the FST, C57/B6 mice display much higher immobility times than 129/SV mice (Lucki et al. 2001). This does not explain the current FST results, because WT mice were more mobile than KO mice. If a background strain effect were indeed

responsible for the obtained results, it would be expected that WT mice would be less mobile in the FST and more mobile in the locomotor test area than KO mice due to a higher proportion of C57/B6-derived genes. In contrast to the findings in the FST and locomotor tests, the results of the light-dark test are consistent with studies comparing 129 (more similar to KOs) and C57/B6 (more similar to WT) mice (Holmes et al. 2003). Therefore, the anxiety-like phenotype of the BGT1 KO mouse may be background strain-related.

From the strain differences existing between C57/B6 and 129/SV mice, it is clear that the data obtained in the current study should not be over-interpreted. It is possible that the variable genetic background of each mouse significantly contributed to the findings in this study, although the known strain differences between C57/B6 and 129/SV mice are not predictive of the outcomes presently obtained. Nonetheless, in the future, the BGT1 WT and KO mice must be further backcrossed to a pure strain and the experiments repeated. Although hitchhiking genes will remain an issue as discussed above, there will be less genetic variability present than in the current study. Alternatively, more sophisticated genetic tools and a BGT1-specific inhibitor would allow for a more clear determination of a role for BGT1 in CNS disorders.

The BGT1 KO mouse had similar seizure thresholds to those of WT mice in several diverse tests. However, other GABA-related behaviors do suggest a role for BGT1 in the CNS. Future studies involving pharmacological modulation of BGT1 or more sophisticated genetic modulation of BGT1 are necessary. However, the current study describes a potential role for BGT1 in the modulation

of depression, anxiety, and neuropsychiatric disorders with a low risk of motor and cognitive side effects. Further investigation of BGT1 in the modulation of neuropsychiatric disorders is warranted.

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CHAPTER 6

DISCUSSION

Aim and significance

The studies presented in this dissertation were designed to test the hypothesis that BGT1 plays a role in epilepsy, seizure control, and certain behaviors, including depression, anxiety, pain, cognition, and motor activity. This hypothesis was based upon several findings: 1) BGT1 transports both the major inhibitory transmitter in the mammalian CNS, GABA, and the organic osmolyte, betaine (Lopez-Corcuera et al. 1992). Both inhibitory tone and osmotic balance play a role in controlling neuronal excitability, making BGT1 a potential target for modulation of excitability; 2) EF1502, an equipotent inhibitor of GAT1 and BGT1, displayed synergistic anticonvulsant action in the absence of increased toxicity when combined with TGB, a GAT1 selective inhibitor (White et al. 2005; Madsen et al. 2009). Synergism is most easily explained by the existence of two functional mechanisms of action, i.e., both GAT1 and BGT1 inhibition; 3) EF1502 reduced the frequency of spontaneous bursting in brain slices held in a hyperexcitable medium, while TGB failed to produce a similar response. Hence, it was suggested that this effect was related to BGT1 inhibition by EF1502 (Smith et al. 2008); 4) several neurological conditions are influenced by GABAergic modulation, including pain, anxiety, depression, and other neuropsychiatric

disorders (Benes and Berretta 2001; Jasmin et al. 2004; Kalueff and Nutt 2007). To address the hypothesis that BGT1 is involved in epilepsy, we determined whether BGT1 mRNA expression was altered in a model of temporal lobe epilepsy, which may suggest engagement of this target in epilepsy. To determine if BGT1 is involved directly in seizure control and behavior, both a pharmacological inhibitor of BGT1 and a BGT1 knockout mouse were utilized. Studies to determine a direct role for BGT1 in acute seizure control involved the determination of the seizure threshold of BGT1 WT and KO mice using four different seizure models. Behavioral characterization of the BGT1 KO mouse included models of depression, anxiety, pain, motor coordination, and locomotion. *In vitro* testing included long-term potentiation determinations in freshly isolated hippocampal slices obtained from BGT1 KO and WT mice. Results obtained from this approach and described in this dissertation provide insight into the potential involvement of BGT1 in epilepsy and several other CNS disorders, and lend support to the hypothesis that BGT1 modulation may be useful for the clinical treatment of neurological disorders.

Summary and conclusions

Chapter 2 describes the temporal expression pattern of BGT1 in the hippocampus following SE. The patterns of other GATs and osmoprotective genes were also determined and compared to those of BGT1. In the pilocarpine SE model, mRNA expression was upregulated early post-SE along with other osmolyte transporters, but was downregulated along with the GABA transporters

at later time-points. These results suggest that BGT1 may possess functionality as both a GABA and an osmolyte transporter in the CNS. It was determined that TonEBP does not exert full control over the expression of BGT1 and the other osmoprotective genes in the brain. TonEBP also does not induce osmoprotective gene expression in response to inflammation, despite its upregulation by an inflammatory stimulus. This is consistent with results obtained in studies performed outside the CNS (Atta et al. 1999; Trama et al. 2000). The results from the current dissertation are in agreement with and expand upon several observations in the literature that suggest that TonEBP is not the major transcription factor responsible for the expression of osmoprotective genes in the brain: 1) TonEBP displays differential expression patterns in the brain compared to the osmoprotective genes with regard to cell type, region, and expression level (Maallem et al. 2006a); 2) TonEBP and osmoprotective gene expression vary by tissue (Zhang et al. 2003); 3) TonEBP is only present and hypertonic-inducible in neurons, while other neuronal cells display upregulation of the osmoprotective genes in response to osmotic stress (Loyher et al. 2004; Maallem et al. 2006b). Dehydration and inflammation do not account for the BGT1 alterations seen post-SE; however, inflammation may contribute to the downregulation of GAT1 and GAT3. These results are again in agreement with previous findings suggesting that GAT1 is modulated in the CNS by inflammation (Wang et al. 2008). Although the molecular regulation of BGT1 in the brain remains unknown, this dissertation determined that BGT1 is altered by SE in a manner similar to both GATs and osmolyte transporters. Since both GABAergic inhibition and osmotic balance play

roles in controlling neuronal excitability, further investigation into the role of BGT1 in seizure control was warranted.

The corneal kindled mouse model has been suggested to be a cost-effective model for the determination of the anticonvulsant potential of investigative compounds against partial seizures (Matagne and Klitgaard 1998). However, the use of this model for the pharmacological characterization of a compound had not been fully validated. The results from this approach are described in Chapter 3. ED_{50}s for nine AEDs, as well as six investigational compounds, were obtained in the corneal kindled mouse model of partial seizures as well as in the MES, 6 Hz, scPTZ, and rat hippocampal kindling models. Results obtained in the corneal kindling model demonstrated positive correlations with those attained using established preclinical models. Hence, the corneal kindling model represents a sensitive and valid preclinical screening tool for the identification of anticonvulsant compounds.

In Chapter 4, the corneal kindling model, along with the 6 Hz, MES, scPTZ, and Frings mouse models, were utilized to determine the pharmacological profile of a BGT1 specific inhibitor, Hit 8. Hit 8 was identified as a specific BGT1 inhibitor using a library screening method (Bolette Christiansen, unpublished). This method was utilized because structural-based methods to identify inhibitors may predispose to identifying compounds that are nonspecific due to a well-conserved substrate-binding site among the GATs (Beuming et al. 2006). Hence, utilizing substrates as scaffolds often leads to the identification of nonspecific compounds.

Hit 8 was tested for anticonvulsant, antinociceptive, and antidepressant activity because GABAergic modulation is known to be involved in these behaviors. The anticonvulsant profile of Hit 8 was determined to be different from that of TGB, making it potentially useful for the clinical treatment of different seizure types than TGB. Unfortunately, Hit 8 has a very narrow therapeutic window for the treatment of seizures. However, at doses well below toxicity Hit 8 was efficacious as both an antidepressant and an analgesic compound. Although these results may be expected from a compound that increases GABAergic tone by inhibiting the reuptake of GABA, it was determined that Hit 8 is in fact not a selective BGT1 inhibitor by the National Institute of Mental Health's Psychoactive Drug Screening Program. It was determined that Hit 8 possesses high affinity for several targets of physiological relevance in seizures, pain, and depression. These include receptor subunits from the serotonin, dopamine, muscarinic, and histamine families, as well as the mu opiod receptor. Due to the high affinity of Hit 8 for these targets, it seems improbable that BGT1 inhibition plays a role in the mechanism of action of Hit 8. Without an available BGT1 specific inhibitor, a mouse lacking BGT1 became the only available tool for the investigation of the role of BGT1 in seizures and behavior.

The results presented in Chapter 5 were obtained from mice that were genetically engineered to lack functional expression of BGT1 protein (Lehre et al, unpublished). Results from these BGT1 KO mice were compared to those obtained from WT littermate controls. Using four different models of seizure threshold (minimal clonic, 6 Hz, minimal tonic extension, and i.v.PTZ thresholds),

it was determined that BGT1 is not directly involved in determining acute seizure susceptibility in the current BGT1 KO mouse. This was unexpected and stimulated further investigation into possible developmental compensatory mechanisms in the BGT1 KO mouse. No upregulation of other GABA transporters or osmolyte transporters was observed in these mice. However, this does not preclude the existence of other compensatory mechanisms. For example, the GAT1 KO mouse does not display upregulation of GAT3, but does display alterations in GAD65/67, $GABA_B$ receptors, and GABA release characteristics (Jensen et al. 2003; Xu et al. 2007; Bragina et al. 2008). With no obvious compensation in the BGT1 KO mice, more subtle behavioral phenotypes warranted investigation. It was determined that the BGT1 KO mouse is not different from WT mice in long-term potentiation or motor coordination. Plasma osmolality was also not different between genotypes, ameliorating the concern that global alterations in osmolality between the two genotypes were masking differences in seizure threshold. For example, BGT1 KO mice were expected to have a higher seizure threshold than WT mice due to an expected increase in extracellular GABA. Since no difference in seizure threshold was ascertained, it was a concern that KO mice may have systemic hyposmolality, which would cause cell swelling in the CNS and mask an increased seizure threshold in the KOs by increasing excitability. Plasma osmolality measurements negated this concern.

Several differences were demonstrated between the WT and BGT1 KO animals. The BGT1 KO mouse may have a pain-related phenotype, although

conflicting results were obtained in the different preclinical models utilized. Since each nociceptive test signals through a different pain pathway, conflicting results may be expected, but must be confirmed with the performance of each test in naïve animals. It was presumed that the writhing test would determine a difference between genotypes if one existed, since it is the most sensitive pain test (Hendershot and Forsaith 1959; Collier et al. 1968). The BGT1 KO mouse appears to have a depressive-like phenotype in the FST that is not explained by possible strain differences, because 129SV mice swim more than C57/B6 mice (Lucki et al. 2001), and KO mice possess a higher proportion of 129SV genes than WT mice. The FST results are also not explained by general hypoactivity of the BGT1 KO mouse. In fact, BGT1 KO mice displayed basal hyperlocomotion that was attenuated by lithium, and not further increased by CDZP/dAMPH. The locomotor profile of the BGT1 KO mouse is not consistent with what would be predicted if strain differences were contributing to the observed results, because 129/SV mice are less mobile under basal conditions than C57/B6 mice, and dAMPH-induced locomotion is not attenuated by 100 mg/kg lithium in 129 mice (Gould et al. 2007), and KO mice possess a greater proportion of 129SV genes compared to WT mice. The BGT1 KO mouse was determined to possess an anxiety-like phenotype in the light-dark box. This phenotype is consistent with what would be expected from the respective background strains of the WT and KO mice (Holmes et al. 2003). The observed depressive and anxiety-like behavior and hyperlocomotion of the BGT1 KO mouse suggest that BGT1 inhibition may produce undesirable side effects, despite the finding that cognition

is not expected to be altered based on the observed LTP results. This particular phenotype of the BGT1 KO mouse raises possible concerns regarding the future clinical utility of BGT1 inhibition if indeed the mouse FST accurately models human depression. To this point, a BGT1 agonist may be more useful clinically. In addition, the BGT1 KO mouse may potentially represent a genetic model of depression, anxiety, and/or neuropsychiatric disorders such as bipolar disorder and schizophrenia given the observed profile in the preclinical animal models utilized in this dissertation research. However, as discussed in Chapter 5, the results obtained with the current BGT1 KO mouse must not be over-interpreted due to several drawbacks of the technology and the possible significant effect of strain differences that exist between KO and WT mice. Nonetheless, the depression and locomotion results obtained were not consistent with those predicted by the responses of the wild type C57/B6 and wild type 129/SV mice, and do support the hypothesis that BGT1 plays a functional role in the CNS. The results from this dissertation provide evidence for continued investigation of BGT1 in CNS disorders.

In summary, although BGT1 is not involved in the control of acute seizure activity, it appears to be involved in epilepsy as evidenced by alterations post-SE that are similar to both osmolyte and GABA transporters. BGT1 is also potentially involved in the modulation of GABAergic behaviors such as pain, depression, anxiety, and neuropsychiatric disorders involving a manic phase, such as might be seen in bipolar disorder and schizophrenia. Further

investigation into BGT1 in both epilepsy and other disorders of the CNS is clearly warranted.

Speculations and future directions

BGT1 is an organic osmolyte transporter that contributes to osmoregulation in the brain. Seizure activity produces an ionic imbalance (Schwartzkroin et al. 1998), and it is possible that an animal lacking the proper regulatory mechanisms to adapt to ionic alterations may have a reduced capacity to recover from such an insult. In the absence of the ability to modulate betaine uptake to compensate for ionic flux, cellular function would degrade and possibly lead to death following an increase in the intracellular ion concentration in BGT1 KO mice (Petronini et al. 1993; Burg 1994; Haussinger 1996). Hence, mice lacking BGT1 may experience increased cell death due to a reduction in the ability to efficiently regulate osmolality. On the other hand, if cell survival is unaffected, mice lacking BGT1 may have reduced astrogliosis if intake of water through BGT1 contributes to cell swelling post-SE (Zhu and Ong 2004).

The results obtained in Chapter 5 suggest that BGT1 may play a functional role in the CNS and support putting forth the investment necessary to develop tools that will allow further study. Although a purely selective pharmacological modulator would be of utmost utility, it has been so far impossible to obtain this. Therefore, several improvements are possible utilizing the genetic approach. The current BGT1 KO mouse could be bred onto a pure background to minimize strain effects. However, as discussed in Chapter 5, hitchhiking donor genes will

still be present (Crusio 1996). Furthermore, the C57/B6 strain may be a poor choice for a genetic background (Mogil et al. 2001). A conditional CNS knockout would perhaps be more useful (Gu et al. 1994), and may reduce the degree of developmental compensation; since osmoregulation is integral to sustain life and the osmoregulatory systems are extremely redundant (Yancey et al. 1982), BGT1 will most certainly be compensated for in a conventional knockout. A better solution to compensation is an inducible KO, in which BGT1 will be ubiquitously expressed until an agent is administered that ablates its expression (Kuhn et al. 1995). Interfering RNA (RNAi) technology may also be useful (Tabara et al. 1998). Here, RNAi could be directly injected or targeted to the CNS to reduce the expression of BGT1 (Mathupala 2009). In addition to genetic knockdown of BGT1, overexpression may also provide valuable information (Zhu et al. 1993).

As discussed in Chapter 2, little is known of the regulation of BGT1 in the brain. In the future, the development of a specific BGT1 antibody with a low detection limit that could identify BGT1 in the CNS would be advantageous for a number of reasons. This would finally mitigate the debate regarding the exact localization patterns of BGT1 in the brain. Furthermore, it would allow the regulation of BGT1 in the CNS to be investigated directly. As discussed in Chapter 2, TonEBP does not appear to exclusively control BGT1 expression in the brain. It is possible that alternate transcription factors are binding to the TonE sites in the promoter region of the BGT1 gene. It is also possible that other promoters are responsible for the control of BGT1 expression in the brain. Eight BGT1 isoforms exist, which differ in their 5’ untranslated region and tissue

distribution suggesting that different promoters may be involved in a tissue-specific manner (Takenaka et al. 1995). Hence, it is possible that a promoter distinct from TonE1 and 2 exists and is functional in the brain. BGT1 tagged with enhanced green fluorescent protein could be transfected into neurons and glia *in vitro* to determine the trafficking and subcellular distribution of BGT1, similarly to what has been done in kidney cells (Kempson et al. 2003). Furthermore, if a specific antibody were available, co-immunoprecipitation studies could be performed to elucidate what BGT1 is binding to in the CNS under conditions of upregulation. *In utero* electroporation techniques could be utilized to tag and investigate BGT1 *in vivo* (Shimogori and Ogawa 2008).

The function of BGT1 in the brain is also unclear. Since it appears from behavioral studies that BGT1 may be involved in the modulation of depression and neuropsychiatric disorders, it may be of interest to investigate the effect of BGT1 knockdown on systems known to be involved in these disorders. For example, the GABAergic system exerts effects on the dopaminergic and serotonergic systems (Soubrie et al. 1981; Nestler et al. 2001); it is possible that levels of these transmitters or of the transporters for these molecules are altered by BGT1 activity. Four general possible functions of BGT1 in the CNS were suggested in Chapter 5.

1) Osmotic or GABAergic regulation. To assess whether BGT1 is functionally a betaine transporter, a GABA transporter, or both, *in vivo* in the CNS, measurements of these substrates could be taken before and after knockdown of the gene using microdialysis. Although plasma osmolality was not different

between BGT1 KO and WT mice, osmolality of the brain's interstitial fluid may be different and may be measured from the dialysate. Electrophysiological studies would provide useful information regarding the effect of BGT1 on inhibitory and excitatory neurotransmission. Unfortunately, it is not expected that the BGT1 KO mouse has the ability to elucidate whether BGT1 plays a significant osmotic role in the brain because the system is extremely redundant (Yancey et al. 1982), and because betaine is not considered one of the major osmolytes in the brain (Gullans and Verbalis 1993). Hence, the effect of BGT1 may not be observable unless the other osmotic systems are blocked. This does not preclude, however, that betaine may indeed contribute to the overall osmotic balance in the brain, a fact that is supported by a reported increase in brain levels of betaine following systemic salt-loading (Lien et al. 1990).

2) GABA and/or betaine transport across the blood-brain barrier. The development of an adequate antibody would strengthen the argument that BGT1 is located at the BBB (Takanaga et al. 2001), from where it would be uniquely positioned to participate in the flux of GABA (Zhang et al. 1999; Kakee et al. 2001). BGT1 is also a likely homeostatic regulator of betaine levels in the brain due to its localization at the BBB. Regulation of betaine uptake and efflux through the BBB allows the brain to be the only tissue in which betaine levels are below plasma levels (50 μM in brain vs 190 μM in plasma in rats) (Slow et al. 2009). Betaine levels are increased in the brain following salt-loading (Lien et al. 1990), and pediatric patients treated with betaine for longer than 4 weeks have been reported to experience cerebral edema (Yaghmai et al. 2002; Devlin et al. 2004).

Although this edema may represent uptake by BGT1, it may also reflect the osmotic opening of the BBB (Kroll and Neuwelt 1998) and successive betaine entry. Nonetheless, the current evidence suggests that betaine levels in the brain are dynamically controlled, very likely by BGT1. Although it is believed that betaine in the brain predominantly originates from the plasma, betaine can be synthesized in the brain through choline oxidation (Katz-Brull et al. 2005). It can also be transported by amino acid transport system A, which is present at the BBB (Petronini et al. 2000; Smith 2000). Previous studies have determined BBB transport of GABA, excitatory amino acids, and taurine (Tamai et al. 1995; Hosoya et al. 1999; Kakee et al. 2001). Similar studies in a controlled *in vitro* expression system could be utilized to determine whether BGT1 is the major transporter responsible for the BBB efflux of betaine.

3) Redistribution of betaine following osmotic stress and/or the redistribution of GABA from GABAergic to glutamatergic neurons. To assess if BGT1 possesses the ability to redistribute betaine in the brain following osmotic stress or seizure activity, methods similar to those employed to determine the redistribution of taurine could be utilized (Nagelhus et al. 1993). It has recently been suggested that BGT1 contributes to the redistribution of GABA from GABAergic to glutamatergic neurons (Sunol et al. 2010). The availability of the BGT1 KO mouse provides an integral control that would greatly strengthen conclusions regarding the contribution of BGT1 to redistribution of substrates.

4) Functionality as a neurotransmitter-gated ion channel. Electrophysiological methods similar to those utilized to determine that GAT1, EAAT4, and EAAT5

can behave as “neurotransmitter-gated ion channels” (Fairman et al. 1995; Sonders and Amara 1996; Arriza et al. 1997; Bagley et al. 2005; Wersinger et al. 2006) could be employed to ascertain a similar function for BGT1 if a specific pharmacological inhibitor for BGT1 were developed. This ion channel function may be particularly relevant for BGT1, which transports three sodium molecules per transport cycle as compared to two sodium molecules transported by GAT1 (Matskevitch et al. 1999). Hence, several potential functions of BGT1 in the CNS remain to be investigated.

The results presented in this dissertation are the first to demonstrate alterations in the molecular regulation of BGT1 in the brain (as well as the osmoprotective genes and TonEBP) following seizure activity and inflammation. Furthermore, it provides the first body of evidence that supports a role for BGT1 in the CNS obtained in a BGT1 KO mouse. The studies contained herein have significantly contributed to our understanding of BGT1 mRNA expression in the CNS, and have elucidated several CNS disorders that BGT1 may be involved in. Although BGT1 does not appear to be involved in the acute regulation of seizure threshold, it does appear to be involved in the chronic condition of epilepsy. Moreover, BGT1 appears to play a potential role in pain, depression, anxiety, and other neuropsychiatric disorders.

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